INTENSIVE PRODUCTION OF *Artemia franciscana* CYSTS IN OUTDOOR PONDS

by

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ACKNOWLEDGMENTS

The project was undertaken at Dampier Salt Limited at Dampier in the NW of Western Australia in the period 1989 to 1994 and was made possible by the full support of the company who provided all facilities, equipment and resources.

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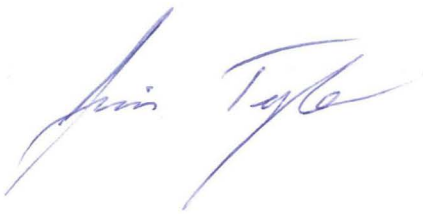

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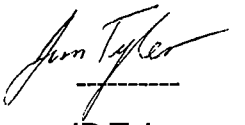

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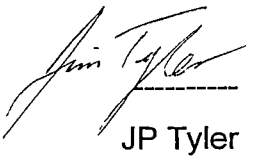

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ABSTRACT

Despite an identified need world wide for reliable supplies of high quality Artemia *cysts for aquaculture, the commercial supply of cysts remains exclusively sourced from unreliable and low productivity extensive systems producing cysts of variable quality. An intensive cyst production system for producing high quality cysts in large outdoor ponds was developed and tested at a commercial saltfield in the North West of Western Australia. To assist in the development of this system numerous experiments were conducted to optimise algal production, identify food requirements of* Artemia, *and spare algal inputs through the use of wheat pollard. In addition, management strategies were assessed for reducing intraspecific competition in order to increase cyst output.*

*Culture conditions for three species of naturally occurring brine microalgae (*Tetraselmis suecica, Dunaliella viridis *and* Dunaliella salina *suitable as food for* Artemia franciscana, *were investigated (Chapter 3).* T.suecica *grew fastest in seawater (density 1.025 g/mL) and was completely inhibited in concentrated brines which are optimal for* A.franciscana *culture (>1.10 g/mL, Chapter 6).* D.salina *grew best at brine densities >1.10 g/mL (Chapter 3.3) and* D.viridis *in less dense brine (<1.10 g/mL). A concentration of 0.01 g/L of soluble fertiliser was found to be sufficient for both* Dunaliella *species in outdoor culture.* T.suecica *had lower productivity (cells/mL/day) than the two* Dunaliella *species by about two orders of magnitude. Four brands of commercially available soluble fertilisers were tested and found to be similar in their effects on microalgal productivity. The optimum physical requirements of both species of* Dunaliella *were similar. Vigorous aeration and peak light intensities above 7,000 lux at noon were found to be necessary to sustain these cultures. Peak* D.viridis *populations (>3 million cells per mL) with a sustainable daily harvest rate >40% of culture volume were achieved. The major problem encountered with outdoor* Dunaliella *culture was grazing pressure from contaminating* A.franciscana *and the brine ciliate* Fabrea salina. *The most effective control procedure for these organisms was found to be; initial sterilisation of culture brine with at least 10 ppm of active chlorine, inoculation with zooplankton-free microalgae culture, continuous screening for A.franciscana, and maintenance of optimal conditions for microalgae culture. This brine often contained* F.salina *which would quickly denude a microalgae pond if allowed to colonise that pond. However, if the brine was added to the A.franciscana pond first, the brine shrimp would effectively eliminate the ciliates before they reached the algal pond.*

The growth responses and conversion efficiencies (dry weight food/dry weight Artemia*), of both solitary and communal A.franciscana, for various amounts of* D.viridis *and* D.salina*, were determined in laboratory experiments (Chapter 4.1). Food level had a marked effect on both growth rates and efficiency at all stages from Instar 1 through to Instar 16 adults. A minimum of between 200,000 and 500,000* Dunaliella *cells per animal per day (0.014 g - 0.035 g dry weight) was required for long term survival of A.franciscana and one million cells per day to enable development through to Instar 16. Maximum growth rate was achieved with four million cells per animal per day. Growth efficiency decreased with increased feeding level. A feeding rate of between one million and two million cells per animal per day was considered to be an appropriate balance between growth and efficiency enabling full animal development with growth efficiency of about 25%. Under highly competitive conditions with uncontrolled* A.franciscana *population growth, the population stabilised at a level where each individual received about 500,000 cells per day.*

Microalgal cultures are relatively dilute on a dry matter basis, however, this is not a limitation with inputs of dry feeds. Finely ground wheat pollard flour supplemented with some D.salina *culture was an adequate food for laboratory cultured A.franciscana (Chapter 4.2). Apparent growth efficiencies for wheat pollard were only about 5% of those achieved with a* D.salina *diet but it was relatively inexpensive. Pollard on its own was also found to be a suitable food for* A.franciscana *in 1,000 L outdoor tanks, however, supplementation with* D.salina *(5% of the total food on a dry weight basis) greatly enhanced the biomass of A.franciscana compared to treatments without supplementation.*

Cyst production relies not just on biomass but on reproductive output and specifically on Artemia *adopting an oviparous reproductive strategy. A minimum food ration of two million* D.salina *cells per individual* A.franciscana *per day was required for high reproductive output. This was difficult to sustain in a communal population due to population increase and resultant increase in intraspecific competition. A number of strategies to limit population expansion were trialed. Selective screening was the one practical technique that was effective in limiting population expansion and maximising reproductive output at a peak of about 25 offspring per female per three day reproductive period. A mesh size of 1 mm or smaller was required to retain physically small broodstock (<1 cm length) in a system with high flow rates. A screen size as large as 2 mm would retain larger adults (>1 cm length) if the flow rates were very low (<500 L/m^2/hour).*

The variety of factors that could conceivably affect the mode of reproduction were investigated (Chapter 5). Laboratory cultured animals tended to be ovoviviparous and animals in outdoor culture oviparous but the switching mechanism was not identified despite extensive experimentation. Food level, salinity, salinity-shock, light intensity and oxygen stress in the presence of iron were all tested and no clear patterns emerged in either laboratory or outdoor trials. Reproductive mode in the 0.08 ha experimental pond was primarily oviparous.

The above data were used to design an operational large-scale facility for the production of A.franciscana cysts (Chapter 6). Major practical problems encountered in the development of this facility included unreliability of electrical equipment in a saltfield environment, inadequate sealing of screens separating the D.salina *pond from the* A.franciscana *pond and, sustaining the population of* D.salina *for long periods. These problems were all largely overcome and cyst production levels in excess of 1 kg per day were achieved from a 0.08 ha pond for several weeks.*

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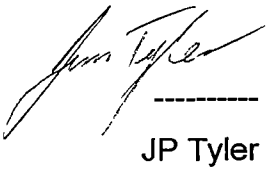

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INTRODUCTION

The demand for Artemia cysts has increased in recent years as a result of a world expansion in marine aquaculture, particularly for prawns, and currently exceeds 1,000 tonnes per year (Bengsten et al. 1991; Van Stappen and Sorgeloos, 1993) with a price of US $85/kg for high quality cysts (Bengsten et al. 1991). Artemia nauplii are a major and preferred source of food for cultured fish and crustacean larvae (Bardach et al., 1972; Goodwin, 1976; Kinne and Rosenthal, 1977; Sorgeloos et al., 1986). The total value of Artemia products exceeds US$50 million per year (Van Stappen and Sorgeloos, 1993). The demand for Artemia cysts is expected to increase at a rapid rate as aquaculture continues to expand world wide.

A history of the supply and demand for *Artemia* has been produced by Bengtson et al. (1991). They describe how a severe world shortage of *Artemia* cysts in the mid-1970's was a major impediment to the expansion of world aquaculture. This lead to a massive research and development programme into *Artemia* in the early 1980's largely centred at the *Artemia* Reference Centre at Ghent University in Belgium. The present world wide cyst production originates from large-scale, uncontrolled, extensive culture conditions and largely meets demand. Quality is, however, highly variable and there is an urgent need for a larger diversity of commercial sources because over 70% of all marketed cysts originate from the Great Salt Lake in the United States. These are considered low quality because nauplii are large (small nauplii are preferred for prawns) and have low levels of essential fatty acids. They are priced at about US$25/kg compared to the US$85/kg price for cysts with high yield, synchronous hatch rates and which produce small nauplii rich in essential fatty acids from pollution free environments.

A large-scale intensive culture system for the production of *Artemia* cysts would ensure substantial and predictable quantities of much needed, high quality product to be produced. It would also eliminate the substantial risk to salt production that can occur with attempts to combine *Artemia* and salt production (Burnard and Tyler, 1993; Tyler and Maguire, 1994). This is discussed in Chapter 1. The need for such a system is especially important for Australia which imports most of its *Artemia* cysts from the United States at a price of up to A$100/kg. The sparsely populated north-west of Western Australia has no intensive agriculture and is very low in pesticide contamination which is a major problem for many sources of *Artemia* cysts (Bengsten et al. 1991; Morrissey, 1993).

This thesis encompasses the development of a large-scale intensive outdoor culture system for the commercial production of *Artemia* cysts

at Dampier Salt Limited in the north-west of Western Australia. Such systems have not been commercially significant despite the large amount of published information on practical aspects of *Artemia* culture (Sorgeloos et al., 1986; Browne et al., 1991). *Artemia* produce either live young or cysts depending on culture conditions but the switching mechanism has not been identified adequately (Gajardo and Beardmore, 1989) despite a large research effort into this aspect (Versichele and Sorgeloos, 1980; Balasundaran and Kumuaraguru, 1987; Berthelemy-Okazaki and Hedgecock, 1987; Gajardo and Beardmore, 1989). Total fecundity has also been well studied with genetic (Browne, 1980; Vanhaecke and Sorgeloos, 1983; Lenz, 1987); environmental (Berthelemy-Okazaki and Hedgecock, 1987; Lenz, 1987; Wear and Haslett, 1987) and nutritional factors (Lavens and Sorgeloos, 1987b; Lenz, 1987) all affecting reproductive output. Mode of reproduction in combination with total fecundity will determine total cyst production but there is little information on practical systems for producing cysts (Bengsten et al., 1991; Tackaert and Sorgeloos, 1991).

The use of microalgae as a food for *Artemia* is considered economically unrealistic for small-scale intensive *Artemia* culture (Lavens and Sorgeloos, 1991). This may not, however, be the case for large-scale culture because the scale of production has the most decisive effect on the cost of unit product in microalgae mass culture (Richmond, 1986b). The use of readily available preconcentrated brine containing many of the nutrients needed by brine microalgae is another factor not generally considered but this will also reduce the production costs. Environmental and nutritional aspects of an intensive culture system need to be assessed in order to ensure maximum production of high quality cysts. The aims of the study are to:

1. Develop a large-scale culture system for the mass production of microalgae food supply.

2. Investigate the growth response and efficiency of *Artemia* to mass cultured microalgae and a suitable inert food.

3. Determine the reproductive response of *Artemia* to a range of conditions including food type and quantity and physico-chemical conditions.

4. Develop population control systems for communal culture to ameliorate adverse effects of intra-specific competition.

5. Design and evaluate a large scale cyst production system.

CHAPTER 1: REVIEW OF LITERATURE

1.1 Morphology and Development

Artemia are members of a primitive order of filter feeding planktonic crustaceans called the Anostraca. They inhabit saline water bodies throughout the world, including solar salt fields, and live for a period of about four months (Sorgeloos et al., 1986).

The development of *Artemia* from a Instar 1 nauplius (Figure 1.1) through 15 moults to fully mature adults (Figure 1.2 and 1.3) has been described by Schrehardt (1987). Females have conspicuous lateral egg sacs (Figure 1.2) and males have overdeveloped antennae called claspers (Figure 1.3). The gradual replacement of the relatively inefficient larval filtration apparatus of antennae and mandibles (Figure 1.1) with the adult thoracopods (Figure 1.2 and 1.3) greatly increases the filtration efficiency of *Artemia* and ensures that adult animals will out-compete their offspring when food is limiting (Schrehardt, 1987). This maximises the chances of adult survival, and the production of more offspring has implications for communal culture systems containing *Artemia* at all stages of development. *Artemia* are relatively non-selective filter-feeders, using the setular mesh of the trunk limbs (thoracopods) to sift bacteria and algae of size 3 to 50 µm from the water column (Reeve, 1963c; Persoone and Sorgeloos, 1980).

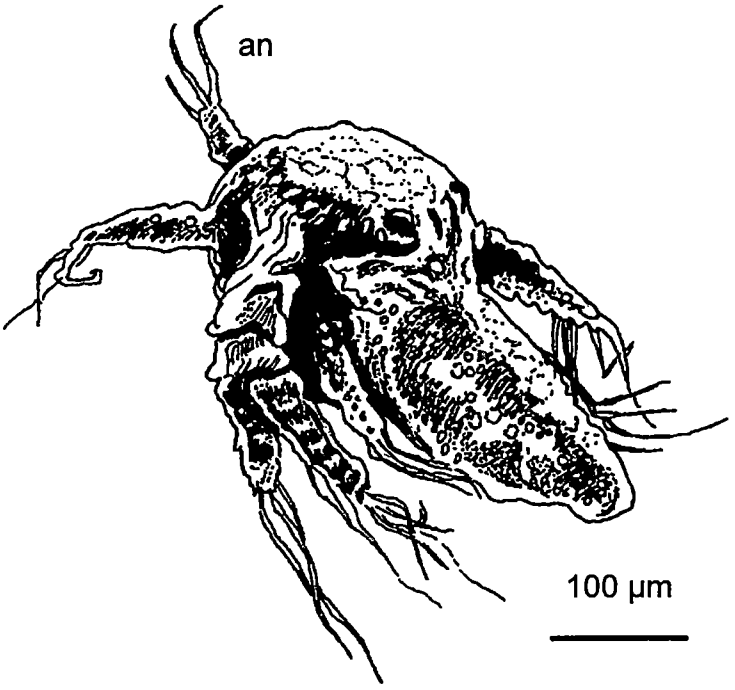

Figure 1.1: *Artemia* nauplius showing antenna (an) used in filtration. After Schrehardt (1987).

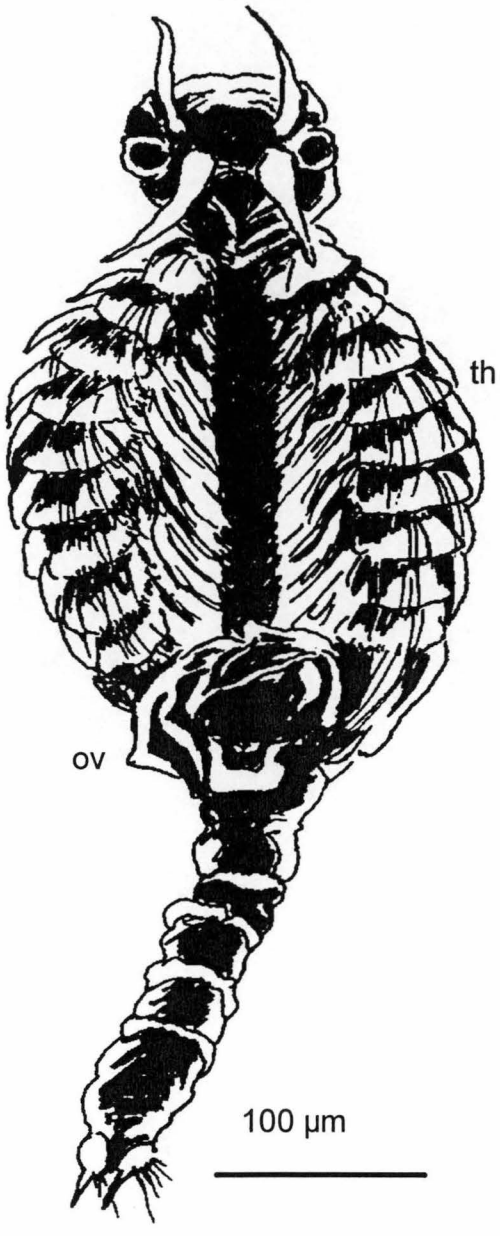

Figure 1.2: Female *Artemia* showing ovisac (ov) and thoracopods (th) used in filtration.
After Schrehardt (1987).

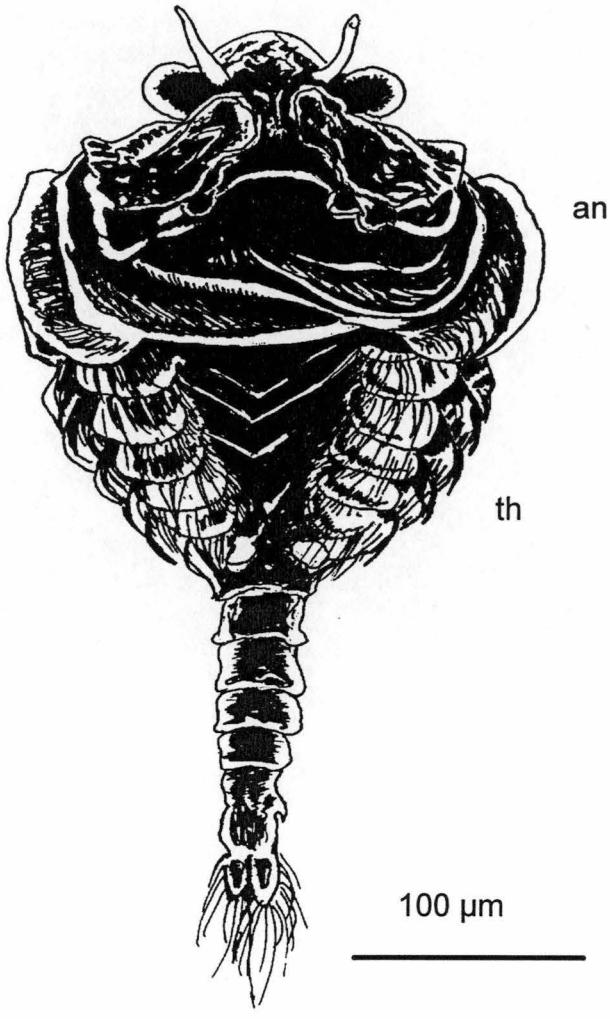

Figure 1.3: Male *Artemia* showing enlarged antenna (an) used as claspers and thoracopods (th). After Schrehardt (1987).

1.2 Role of Artemia in Solar Salt Production

Semi-intensive production of *Artemia* in permanent saltfields has been reviewed by Tyler and Maguire (1994). A consideration of the role of *Artemia* in solar salt production is relevant because it clarifies why the integration of commercial *Artemia* culture and salt production from permanent, rather than seasonal saltfields, cannot be pursued without risk to salt production. This in turn increases the relevance of an intensive culture system separate from the saltmaking process, as developed in this thesis.

Solar salt is produced by the stepwise concentration of seawater through a series of increasingly saline ponds ultimately resulting in

the deposition of salt in crystallisers. The phase chemistry of saltmaking (Baseggio 1974, Appendix 1) and saltfield design (McArthur,1979) are important factors. The biological system of a large solar saltfield, operated by Dampier Salt Limited at Dampier (20.39^{o} S 116.45^{o} E) in the north-west of Western Australia (Figure 1.4) and producing over three million tonnes of salt per year, has been well documented (Sammy, 1984; Burnard and Tyler, 1993). There is a decrease in species diversity with increasing salinity resulting in large communities of fish and seaweeds in the early ponds being gradually replaced by carpets of microbial mats, dominated by slime-producing unicellular cyanobacteria, and planktonic *Artemia*.

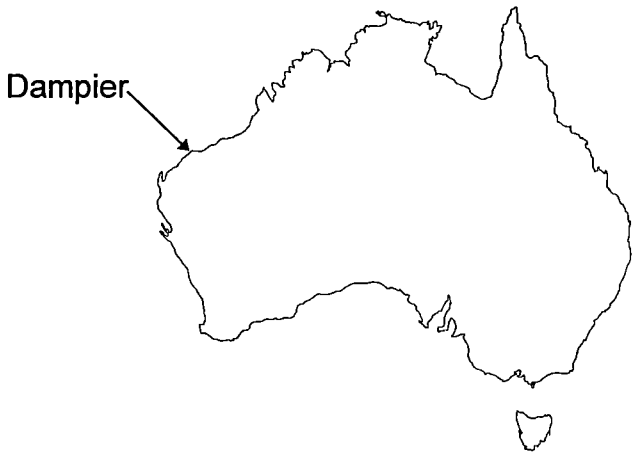

Figure 1.4: Location of Dampier Salt in the NW of Western Australia (20.49^{o}S, 116.45^{o}E).

The role of *Artemia* in the production of solar salt has until recently been misunderstood. *Artemia* clear the water column and enable high levels of light to reach the floor of the pond. This in turn imparts stability to benthic microbial mats and prevents the long chain polysaccharide molecules (>20,000 Daltons) contained within the mats from dissolving in the brine (Roux, 1992; Burnard and Tyler, 1993). These polysaccharides elevate brine viscosity and ultimately disrupt both salt production and product quality (Burnard and Tyler, 1993; Tyler and Maguire, 1994). This means that usefulness to the saltfield of *Artemia* in keeping brines clear will be counteracted by attempts to increase their population via the use of fertilisers which will cause both increased shading and physically stimulate the growth of the potentially harmful microbial mat organisms. This is the major reason why *Artemia* culture should be pursued in ponds separate from the saltmaking process in permanent saltfields. Integration is more feasible is seasonal saltfields which do not develop extensive microbial mats (Tyler and Maguire, 1994).

1.3 Systematic Classification and Genetics.

A review of the taxonomy of *Artemia* is relevant to the culture of *Artemia* because genetic factors influence both mode of reproduction and total reproductive output (Browne, 1980; Vanhaecke and Sorgeloos,1983; Lenz, 1987).

The taxonomy of *Artemia is* difficult because of phenotypic changes in response to variable environmental conditions such as salinity and oxygen levels (Gilchrist, 1956; 1959; Baid, 1959; Abreu-Grobois, 1987). *Artemia salina* Linnaeus was the name generally given to all populations of *Artemia* until Bowen and Stirling (1978) questioned the validity of this species name. Six sibling species were recognised by 1980 (Bowen et al., 1980) and reproductive isolation was confirmed by crossing experiments conducted by Barigozzi (1980). Populations of *Artemia* were studied by Beardmore et al. (1983) using electrophoretic, cytological and anatomical techniques. They also concluded that distinct species do exist. Chromosome studies (Abatzopoulus et al., 1987; Baratelli, 1987; Barigozzi et al., 1987), isozyme patterns (Abreu- Grobois and Beardmore, 1980; Requintina and Simpson, 1987), and haemoglobin structure (De Smet et al., 1987; Wolf et al., 1987) confirm this view. In their comparative study on haemoglobin, De Smet et al. (1987) concluded that the gene duplication to give a current homology of between 25% and 40% must have occurred between 500 and 850 million years ago. A review of the genetics of *Artemia* was published by Abreu-Grobois (1987). Five species are listed.

The species present at Dampier Salt is *Artemia franciscana* and was introduced from two sources. The first introduction was from cysts collected from the Shark Bay saltfield (700 km south of Dampier) in 1973. The Shark Bay saltfield had apparently been inoculated with cysts from San Francisco Bay in 1971 (Dampier Salt internal report). The population of *A.franciscana* in the Dampier saltfield declined to very low levels in 1983 following biological disturbances in the ponds (Section 1.1). This led to a reintroduction of *A.franciscana* hatched from San Francisco Bay cysts (Biomarine Brand) in 1983 and 1984.

A.franciscana is a diploid species in which the sexes are separate. It is found throughout the world in a range of brines of different ionic composition. Ecological barriers separate many populations of this species and incipient speciation is apparent (Bowen et al., 1980). *A.franciscana* from high chloride brines, for example cannot survive in Mono Lake (in California) but can be crossed with Mono Lake *"A.franciscana"* to produce viable offspring (Abreu-Grobois, 1987). Beardmore and Abreu-Grobois (1987) suggested that Mono

Lake *Artemia* merited specific status as *A.monica*. *A.franciscana* adapts very quickly to a new environment. Bernaerts et al. (1987) found that the upper temperature tolerance of *A.franciscana* from San Francisco Bay (35°C) was 10°C lower than that of animals from Macau. The animals used in the experiment were raised under controlled conditions from cysts and the differences were assumed to be genetic and have occurred within two years of separation of the stocks. Genetic adaptation to high temperature was also indicated in a study by Vanhaecke et al. (1984). Browne et al. (1984) identified a divergence in the proportion of encystment in *Artemia* from San Francisco Bay and a population originally from the Bay, but kept in the laboratory for 25 years.

A.franciscana collected from the Dampier Salt ponds was used in all test work for the following reasons:

- It has small cysts (about 200 µm diameter), desirable for aquaculture.
- All previous work at Dampier had been done with this strain.
- Large quantities of cysts were available from the salt ponds.
- It had adapted to Dampier conditions.
- Contamination with the Dampier strain was inevitable if any other strain was chosen.

1.4 Growth and Ecology

An understanding of the growth and ecology of *Artemia* is fundamental to the successful establishment of a large-scale communal culture system. The three main physiological adaptations of *Artemia* to their environment are:

- Ability to tolerate a wider range of salinities than any other animal.
- An ability to tolerate very low levels of oxygen by synthesising haemoglobin as required.
- The production of desiccation resistant resting cysts to enable the species to survive intermittent drying of ponds (Sorgeloos, 1980).

It is this last characteristic that makes *Artemia* so valuable in aquaculture and the primary reason for much of the scientific and commercial interest in *Artemia*.

Growth rates of *Artemia* vary with environmental factors and genetics. Optimum growing conditions have been investigated by Bossuyt and Sorgeloos (1980); and Wear and Haslett (1987). A review paper by Persoone and Sorgeloos (1980) lists the following ecological tolerances although it is likely that the strain of *Artemia* present in the Dampier Salt Limited brine concentrating ponds has genetically adapted to the warm pond conditions at Dampier (up to 35°C in summer) in a similar manner to the Macau animals described by Bernaerts et al. (1987).

Temperature:	6 to 30°C optimum 25 to 30°C
Ionic balance:	High cations: anion ratio
Salinity:	Brackish (fish dependent) to 340 ppt
Oxygen:	I ppm to saturated. Optimum near saturated
pH:	Neutral to alkaline. Cyst hatching inhibited below pH 8.0

It appears that the growing conditions for *Artemia* are not critical and will be dictated in culture more by considerations of cyst production and quality; than by animal tolerance; these aspects are emphasised in this study.

The growth response of various strains of *A.franciscana* to a range of microalgae has been well researched (Reeve, 1963a, b, c; Mason, 1963; Johnson, 1980; Brune and Anderson, 1984; Rowsowski, 1989). *Artemia spp.* are, however, intrinsically highly variable (Abreu-Grobois,1987) and the nutritional value of cultured microalgae can vary greatly with species and culture conditions (Jeffrey and Garland, 1987; Brown et al., 1989). Some authors (Mason, 1963; Johnson, 1980; Rowsowski, 1989) used *A.franciscana* from San Francisco Bay while others (Reeve,1963a, b, and c; Brune and Anderson, 1989, 1984) used *A.franciscana* from the Great Salt Lakes. None of the studies used *D.salina* or *D.viridis* although *D.tertiolecta,* a lower salinity species, was used in some studies (Mason,1963; Johnson, 1980). *D.tertiolecta* grown in low salinity media has, however, been found to be nutritionally inadequate for *Artemia (*D'Agostino and Provasoli (1968). Little is known of the food value of *Dunaliella spp.* from mass cultures in brine. Most of the published studies used axenic laboratory culture, yet the value of bacteria in the diet of *Artemia* has been well documented (Gibor, 1956; D'Agostino and Provasoli, 1968; Douillet 1987). Intriago and Jones (1993) found that bacteria acted not only as food for *Artemia* but assisted in the digestion of the algae.

An examination of the published data reveals great discrepancies. The two major studies on growth efficiencies yielded results

ranging from 4% to 5% (Mason, 1963) up to peak efficiencies of 79% (Reeve, 1963a). The effects of competition on growth and efficiencies are not well understood even though a mathematical simulation has been produced by Brune and Anderson (1989), using the data of Reeve (1963a), to explain size diversity in *Artemia* cohorts. Most published growth studies on *Artemia* used communal animals (Gibor, 1956; Reeve, 1963a,b and c; Johnson, 1980; Rowsowski, 1989) while Mason (1963) used solitary animals for his growth efficiency study.

The published information could not, therefore, be used with confidence to determine optimum feeding rates for Dampier strain *A.franciscana* fed *Dunaliella spp.* mass cultured in outdoor ponds, thus making this a critical area of study.

1.5 Cyst Production and Quality

The identification of factors affecting cyst production is the major focus of this thesis.

Artemia spp. exhibit great flexibility in reproduction. They can reproduce either sexually or parthenogenetically and produce either live young or cysts depending on culture conditions. The ability to switch reproductive mode appears to be related to maternal heterozygosity but the triggering factor has not been conclusively identified (Gajardo and Beardmore, 1989).

Genetic studies have revealed that *A.franciscana is* an ideal species for cyst production. A literature review conducted by Lenz (1987) revealed that environmental and genetic factors affect the production of cysts. Of the five strains compared by Browne (1980), the *Artemia* from San Francisco Bay produced the greatest volumes of cysts (7.8 cysts/female/day) with the highest viability. The Puerto Rican strain was next highest with 4.4 cysts/female/day. Vanhaecke and Sorgeloos (1983), in a comparison of hatching data of ten commercial strains of cysts, found that cysts from San Francisco Bay (SFB) and Brazil (Derived from SFB Stock) had the highest and most synchronous hatch rates. It is likely that the *A.franciscana* at Dampier Salt will also be a good cyst producer. No genetic studies were conducted in the present study.

Versichele and Sorgeloos (1980), and Balasundaran and Kumaraguru (1987) found that *Artemia* subjected to a combination of oxygen stress and high levels of iron (added as Fe EDTA) developed high haemoglobin levels in laboratory experiments. The proportion of cysts as a percentage of total reproduction increased

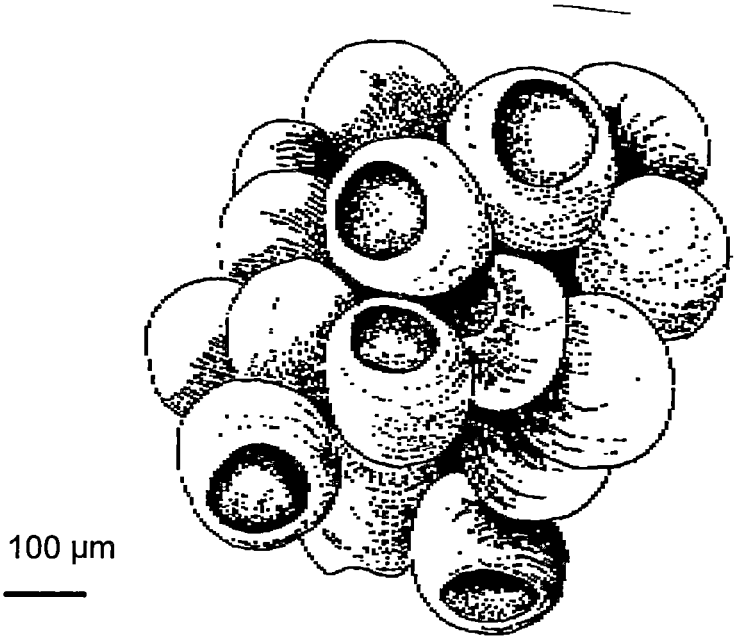

Figure 1.5: Dehydrated *Artemia* cysts showing characteristic indentation

from around 25% to over 90% of total reproduction in these animals. Wolfe et al. (1987) also found that haemoglobin synthesis was stimulated by hypoxic stress although the study did not examine cyst production. Three types of haemoglobin with specific oxygen combining characteristics occur in *Artemia* and the proportions as well as total quantities can change (Wolfe et al., 1987). The mode of reproduction was not related to the specific type of haemoglobin in any of the above studies. The effect of oxygen stress and iron on cyst production from the Dampier strain of *A.franciscana* will be tested in both indoor and outdoor culture as these are factors that can be manipulated on a large scale.

Environmental factors including photoperiod, temperature, salinity and maternal age all had strong influences on reproductive mode in a study by Berthelemy-Okazaki and Hedgecock (1987). They found that the interactions between factors were more important than any factor on its own. The study produced inconclusive results for population density and hypoxia.

Brine salinity is the most easily manipulated environmental factor in large-scale culture and indirectly affects other factors such as oxygen concentration and viscosity. The effect of salinity on cyst production will be a major aspect of this thesis, particularly as there are contradictions in the literature. There are widespread reports

that Artemia are ovoviviparous in low salinity ponds and viviparous in high salinity ponds (Davis, 1980; Sorgeloos et al., 1986; Tackaert and Sorgeloos, 1991) but these reports contradict the experimental findings of Berthelemy-Okazaki and Hedgecock (1987) and my own observations of *Artemia* in the Dampier saltfield over a ten year period. It is likely to be impractical to manipulate photoperiod, temperature and maternal age in large-scale outdoor culture and these topics will not be addressed.

Wear and Haslett (1987) found that fecundity, generation times and reproductive lifespan were enhanced at temperatures between 20 and 28°C and salinities between 100 ppt and 170 ppt in a study of *Artemia* from Lake Grassmere in New Zealand. Food level and quality have an effect on both reproductive mode and total fecundity (Lenz, 1980; Lavens and Sorgeloos, 1987) with higher food levels increasing fecundity. The effect of food quality and quantity is a major study topic in this thesis.

The price of *Artemia* cysts is largely determined by quality with export prices varying from about US$25/kg up to US$80 (Bengsten et al., 1991). However, 100 tonnes per annum of extremely low quality and unprocessed cysts are produced at the Tang Gu saltfield in China and are sold locally for between US$1.28-4.25/kg (Tackaert and Sorgeloos, 1991). The majority of marketed cysts are collected from extensive situations and are highly variable in terms of quality as a result of the uncontrolled conditions under which they are produced (Bengsten et al., 1991). In a more controlled intensive situation the quality of cysts should be much more consistent and a high priority would be placed on quality. High quality cysts are characterised by high yield, synchronous hatching and small nauplii containing high levels of Highly Unsaturated Fatty acids (HUFA) (Bengsten et al., 1991; Lavens and Sorgeloos, 1991).

Hontorio et al. (1989) proposed a model of quality evaluation addressing the following characteristics:

- % of full and empty cysts.
- Dehydrated cyst weight.
- Hydrated cyst diameter and volume.
- Length and dry weight of hatched nauplii.
- Hatching efficiency.
- Hatching rate.
- Synchrony and times of hatching.
- Nutritional value.

Cogwill et al. (1987) evaluated the quality of commercial cysts from three sources and found high variation. Specifically, cysts from San

Francisco Bay were particularly variable and deduced to have originated from several geographical locations. The quantities of components critical for the nutrition of target species, including Highly Unsaturated Fatty Acids (HUFA), Selenium and Manganese, varied greatly (Cogwill et al., 1987). The variability in the quality of commercial cysts is a major problem for buyers and is the result of collecting cysts produced by animals grown in uncontrolled extensive conditions.

The hatchability of produced cysts is a major quality factor and must be considered in an operational system. The culture conditions of the parent animals have a major influence (Lavens and Sorgeloos, 1987). Cysts produced at higher salinities have better hatching percentages than those produced at lower salinities and cysts produced by starving adults also have a better hatch rate than those produced by well fed adults although total production may be reduced.

An important variable in assessing the hatching percentage of *Artemia* is dormancy. Cysts are produced in a dormant state called diapause, with embryonic development halted at the gastrula stage (Cacae et al., 1987; Drinkwater and Clegg, 1991). No development is possible until diapause has been broken. Crowe et al. (1987), give dehydration (anhydrobiosis), cold (cryptobiosis), lack of oxygen (anoxybiosis) as three methods for terminating diapause and this is confirmed in the major review by Drinkwater and Clegg (1991). Methods for diapause deactivation are strain dependent and have been reviewed by both Lavens and Sorgeloos (1987), and Drinkwater and Clegg (1991). The usual method, and one which works well with cysts produced at Dampier is dehydration, either in air or in a brine of density greater than 1.08 g/mL (salinity >107 ppt). When cysts are dehydrated, trehalose replaces water around polar residues in membrane phospholipids and proteins. This enables a fluid state to be maintained in the absence of water (Crowe et al., 1987). Chemicals such as peroxide and hypochlorite can be used to chemically deactivate diapause. Once diapause has been broken the cysts are quiescent and will hatch under appropriate conditions. Quiescent cysts are easily killed, particularly by partial rehydration for too long or by too much. There is a suggestion that secondary diapause can occur in cysts stored under anoxic conditions (Drinkwater and Clegg, 1991) and that this may account for the apparent loss of viability of some stored cysts.

In their manual for the use of *Artemia* in aquaculture, Sorgeloos et al. (1986) describe the hatching of cysts as an osmotic process. Hygroscopic glycerol in the outer cuticular membrane draws water through the permeable cyst shell. The resultant internal pressure splits the shell and enables the nauplius to emerge. Funke and Spindler (1987) describe the hatching process as involving both osmotic and enzymatic mechanisms and similar to the moulting

process in crustaceans. The process is apparently controlled by ecdysteroid hormone levels. Many factors influence the hatchability of cysts (Vanhaecke and Sorgeloos, 1983; Thun and Starrett, 1987; Sawchyn, 1987). Light is essential for the hatching of cysts, a haemopigment located in the gastrula is involved in photoreception and induces the hatching process (Van der Linden et al., 1987). Haematin in the cyst shell tends to shield the light receptor and darker cysts therefore require more light to initiate the hatching process. A wavelength of 400 nm to 600 nm the most effective at inducing hatching.

In their review paper Lavens and Sorgeloos (1987b) suggest the following conditions for optimum hatching:

Temperature:	15 to 30°C. Below -10 and above 40°C is lethal.
Salinity:	Below 85 to 90 ppt. Faster at lower salinities.
pH:	8.0 to 8.5
Oxygen:	2 ppm.
Cyst density:	17 g/L
Light:	26200 microEinsteins/m^2 once fully hydrated

The chemical removal of capsules from the cysts by a process called decapsulation generally improves hatching percentage and speed of hatching (Bruggeman et al., 1980; Vanhaecke and Sorgeloos, 1983) as well as sterilising the cysts. The chemical generally used is hypochlorite. Bruggeman et al. (1980) suggest 15 g of cysts in 200 mL of 70% weight percent activity $Ca(OCl)_2$ but do not specify an optimum time.

Harvesting, processing and storage techniques for cysts are critical in an applied culture system to maximise the harvest of produced cysts and ensure that no deterioration in quality occurs during the processing procedure. The cysts produced naturally in the brine concentrating ponds of Dampier Salt Limited at Dampier are between 200 µm and 220 µm in diameter and float in concentrated brine. There is little in the literature on practical techniques for harvesting cysts. Cysts are generally collected from beaches on the leeward side of ponds containing *Artemia*. A harvesting technique utilising flotation, skimming and possibly screening will need to be developed for large scale cyst harvesting in a commercial system.

Collected cysts need to be dehydrated to <10% and preferably to <5% water and stored in an oxygen free environment for long term storage (Lavens and Sorgeloos, 1987). This can be achieved by air drying, preferably at 40°C before storing under vacuum or nitrogen

at low temperature (Sorgeloos, 1980). Water content is only reduced to around 20% when cysts are placed in a saturated brine (Sorgeloos, 1987) so this is less suitable as a storage medium. Cysts can also be dried to 5% moisture using saturated Magnesium Chloride solution (Sorgeloos et al., 1986). Salt can be quickly washed from cysts using cold fresh water (Sorgeloos et al. 1980). This should not take more than ten minutes and the cysts, held by a 50 µm screen should be physically squeezed to remove as much water as possible prior to drying in air, in a fluidised bed drier, or in an oven at a temperature less than 40°C (Sorgeloos et al., 1986). The metabolism of cysts is inhibited below 4°C (Lavens and Sorgeloos, 1987(b)) so if cysts are treated below this temperature problems of nauplius development within the hydrated cyst are minimised.

The effectiveness of harvesting and storage techniques can be quickly gauged by routine viability tests. The major potential risk in exploitation factors involves rehydration and combinations of dehydration and rehydration cycles (Lavens and Sorgeloos, 1987b). Unlike some other strains of *Artemia,* a single dehydration is sufficient to deactivate diapause in cysts collected from the brine concentrating ponds of Dampier Salt Limited at Dampier (personal testing). Much care should then be taken to avoid consequent rehydration.

1.6 Artemia Culture Systems

A recent review of literature relevant to the semi-intensive culturing of *Artemia* in fertilised ponds has been produced by Tackaert and Sorgeloos (1991) and largely deals with the seasonal production of *Artemia* in small solar saltfarms where some control can be exercised over salinity, water retention time and food availability. This is in contrast to intensive production for *Artemia* biomass based in indoor tank systems under completely controlled conditions as described by Lavens and Sorgeloos (1991). The production of *Artemia* in large permanent saltfields is limited to control of opportunistic dispersion of *Artemia* due to the risks to solar salt production of fertilising the brine and stimulating the growth of undesirable cyanobacteria (Davis, 1979; Davis, 1980; Jones et al., 1981; Sorgeloos, 1983; Sorgeloos et al., 1986; Tackaert and Sorgeloos, 1991; Burnard and Tyler, 1993; Coleman and White, 1993).

An intensive large-scale outdoor culture system for *Artemia* would involve the intensive feeding of animals in large aerated ponds with control exercised over parameters such as food type and level, degree and type of aeration, brine density (g/mL), and rate of

turnover of culture brine. Other parameters such as light intensity and brine temperature can be indirectly controlled by factors such as brine depth; pond orientation and shape; and height of walls. Many of the intensive culture techniques detailed by Sorgeloos et al. (1986) and Lavens and Sorgeloos (1991) may be applicable to a large scale culture system and are a good base for initial applied test work.

The large scale culture system being developed in this study involves a primary food source of mass cultured microalgae possibly supplemented with a suitable inert food. Brine microalgae are the natural food for *Artemia* but some brine species such as the unicellular cyanobacteria *Synechococcus* are indigestible and others, including some dinoflagellates, are toxic (Lavens and Sorgeloos, 1991). The nutritional value of microalgae is another factor to consider and this is greatly influenced by the culture conditions of the microalgae (Jeffrey and Garland, 1988; Brown et al., 1989; Lavens and Sorgeloos, 1991). A primary study area will therefore be the development of an effective culture system of a suitable brine microalga. Despite brine microalgae being the natural food for *Artemia* and the basis of all commercial cyst production from extensive systems, there have been few published studies on the growth and reproductive response of *Artemia* to these microalgae. This will be a major part of the study. The use of suitable inert feeds as substitute or supplementary feeds will also be investigated.

1.7 Mass Culture of Microalgae

Extensively cultured *Artemia* coexist with their food supply and productivity is dependent on the productivity of microalgae within the ponds and, in the case of salt fields, plankton entering with brine from earlier ponds which are free of *Artemia* due to the presence of fish. The high filtering efficiency of *Artemia* generally ensures that most of the microalgae is removed from the brine column and productivity is therefore low.

Intensive culture of *Artemia* involves the addition of food to animals cultured in aerated brine of suitable pH, density and temperature. Food can be microalgae mass cultured in separate ponds; a suitable inert food of particle size 3 to 50 µm (Sorgeloos et al., 1986) or a combination of both.

The growth response of *Artemia* to various types and quantities of food, particularly microalgae, is investigated in Chapter 4. The mass culture of microalgae in brine is fundamental to any intensive culture system utilising microalgae as food.

Dunaliella spp. and *Tetraselmis suecica* are two dominant genera of microalgae suitable as food for *Artemia franciscana* in the Dampier Salt Limited brine concentration ponds with brine of density 1.08 g/mL to 1.14 g/mL (Salinity 107 -180 ppt). The mass culture of these species is the subject of Chapter 3.

1.7.1 *Dunaliella*

The culture conditions for *Dunaliella spp.*, particularly its salinity tolerance (Borowitzka,1974), are well documented because this organism is grown commercially in brine throughout the world for the production of Beta-carotene and glycerol. One species of unidentified *Dunaliella* isolated from Great Salt Lake was studied by Van Auken et al. (1973). The optimum growth conditions which enabled a ten hour doubling time in the laboratory were as follows:

Temperature	32°C
NaCl	19.2%(W/V)
CO_2	1 - 2% at a rate of 2.2 mL/min/mL of culture.
Light Int.	25 - 35 klux
pH	5.8 - 6.5
K+/Na+	< 1, ideally <0.1

Ginzburg and Ginzburg (1981) studied the effect of several environmental variables on the growth rates of several *Dunaliella* strains. They found two distinct groups. A halotolerant group capable of growing at 0.5M NaCl and above, and a halophilic group that required at least 2M NaCl to grow. It was difficult to specify optimal environmental conditions because of integrated effects e.g. optimum temperature was higher at higher light intensity, and ability to tolerate high NaCl levels was enhanced with high light intensities and plentiful carbon.

The species of *Dunaliella* dominating in brine of density less than 1.10 g/mL Dampier Salt Limited is *D.viridis* (using key translated from Massyuk, 1980). *D.viridis* was studied in laboratory culture by Baas-Becking 1931 with the following results.

1. *D.viridis* Teodoresco thrives equally well in solutions of NaCl 1 M to 4 M and pH 6.0 to 9.0.

2. It is sensitive to calcium and magnesium ratios, particularly in acidic conditions.

3. Calcium and Magnesium are antagonistic in their effects with optimal balance dependent on NaCl concentration. In a 1M solution of NaCl the optimal ratio of Mg:Ca is 4:5 and in a 4M solution 20:1. Provasoli et al. (1956) found that the Ca:Mg ratios were critical and antagonistic for two other microalgae species *Rhodomonas lens* and *Phormidium persicinum.*

4. The increase in the antagonistic relationship Mg:Ca in the media in which *D.viridis* live parallel the changes that occur when seawater evaporates.

The mechanism of CO_2 fixation in *D.tertiolecta* can vary during growth in batch culture (Mukerji et al., 1978). Cells in the exponential phase of growth utilised free CO_2 while those in the stationary phase utilised the bicarbonate ion as the substrate for photosynthesis.

D.salina cultures grown at high salinities (15 - 25% NaCl) grew more slowly than those grown at low salinities (Loeblich, 1976). High temperatures and acidic pH produced similar effects. Further study revealed that growth was probably limited by CO_2 in all cases. Dissolved CO_2 gas and CO_2 converted from the carbonate ion through the action of the enzyme carbonic anhydrase are both utilised.

The above information suggests that *Dunaliella* is tolerant of a wide range of conditions. In a culture system utilising fertilised brine, the concentration of CO_2 appears to be critical. A functional system would therefore appear possible using brine fertilised with the two macronutrients likely to be limiting, phosphate and nitrate. CO_2 levels can be maximised by a combination of aeration and relatively high pH (which increases the solubility of CO_2 which in turn as an acid decreases pH). Care must be taken to prevent the precipitation of trace metals by elevating the pH too high. Summer light intensities exceed 20 klux at noon so some degree of shading, if self shading is insufficient, may be necessary. All these aspects will be tested.

1.7.2 *Tetraselmis*

T.suecica is a commonly requested genus from the CSIRO Marine Laboratory culture collection in Hobart Tasmania, Australia (Jeffrey and Garland, 1986). Preliminary studies at Dampier Salt have shown that *T.suecica* (identified by CSIRO

Marine Laboratories in Hobart) isolated from the saltfield is an excellent food for *Artemia* although it does behave very differently in culture to the strain of *T.suecica* obtained from the CSIRO as well as having much larger cells and slower rates of growth in "F media' at 25°C.

Tetraselmis is synonymous with *Platymonas*, the earlier name for this genus. Fogg (1975) reports that *P.subcordiformis* adheres to the walls of culture vessels making turbidostat culture difficult. Maddux and Jones (1964) however, grew *Tetraselmis sp.* successfully in turbidostat culture. The Dampier Salt strain of *T.suecica* adheres to the glass walls of the culture vessels in a similar manner to that described by Fogg (1975). This was also a problem when growing *T.suecica* in 1,000 L fibreglass tanks in preliminary trials at Dampier Salt. Chapman and Chapman (1973) report that *P.subcordiformis* had positive phototaxis with low light and negative phototaxis with high light.

The optimum conditions for growth of *Tetraselmis sp.* are interrelated. Maddux and Jones, 1964, found that nutrient concentration was unimportant at low light intensities but became important as salinity increased. Under conditions of medium light intensity in turbidostat culture, a four fold increase in growth rate was achieved by raising the level of nitrogen (as nitrate) and phosphorous (as phosphate) from 0.125 and 0.012 mg/L to 140 and 155 mg/L respectively (Maddux and Jones, 1964). This implies that nutrient level, even under conditions of nutrient excess is important. The optimum temperature for growth was found to be lower when NO_3 and $P0_4$ concentrations were similar to those found in natural waters than in culture, when higher concentrations were used.

1.7.3 Comparison of requirements of *Dunaliella spp.* and *T. suecica*

T.suecica occurs naturally in the Dampier Salt brine system until a density of 1.10 g/mL is reached and gypsum begins to deposit. This is in keeping with the findings of Carpelan (1964) who found that the ionic changes occurring after gypsum deposition eliminated *Tetraselmis sp.*

D.viridis occurs in brine throughout the Dampier saltfield, but is outcompeted by *T.suecica* in brine of salinity 1.08 g/mL to 1.10 g/mL during summer. The pH, salinity and ionic composition of the brine are constant in each salt pond throughout the year. The dominance of *T.suecica* during the summer period is probably related directly, or indirectly, to changes in physical conditions. Temperatures, light intensities and average daily run

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of wind are all significantly increased during summer (Dampier Salt routine pond monitoring data).

Both *Dunaliella spp.* and CSIRO *T.suecica* have a wide tolerance to temperature (Jeffrey and Garland, 1988) and grow well at all temperatures between 10°C and 30°C.

1.7.4 Nutritional Requirements

The optimum nutrient concentrations are species specific but most species can tolerate high nutrient levels (Kaplan et al., 1986). An excess of required nutrients is, therefore, generally added to the culture media to ensure none are limiting. High nutrient concentrations can sometimes facilitate nutrient uptake and stimulate the growth of algae. An example of this was the fourfold growth response achieved by a tenfold increase in concentrations of nitrate and phosphate mentioned above (Maddux, 1964).

Nutrient levels affect both the final yields and the growth rates. The optimum amount of nutrients for any specific species will vary with the culture conditions, particularly temperature, pH and light intensities (Kaplan et al., 1986).

Macronutrients are substances needed in relatively large quantities (>l mg/L) whist micronutrients are those needed in small amounts (<l mg/L). An explanation of the role of individual nutrients can be found in Vonshak (1986). The three macronutrients likely to become limiting in a brine based culture system are Phosphorous, Nitrogen and Carbon. Micronutrients are less likely to be limiting because all trace metals present in seawater are present in the brine in concentrated form. One possible exception is iron which is precipitates as iron oxide from concentrating seawater (Baseggio, 1972). It appears, therefore, that the addition of Phosphate, Nitrate and Carbon in either pure form, or in a complete commercial mix, to brine should be an effective culture medium. The optimisation of nutrient levels will be a fundamental step in developing an effective microalge culture system.

1.7.5 Culture Conditions

Growing cultures outdoors involves a continuous response of cells to an environment changing diurnally and seasonally. The general effects of environmental factors were reviewed by Richmond (1986a). The three main factors that can be easily

modified in outdoor culture are salinity (affecting osmoregulation and ionic regulation), light intensity and turbulence. Brine pH is also critical and will be affected by factors such as CO_2 concentration in the brine which in turn will be affected by factors such as degree of aeration. These factors are critical in developing an optimum system for microalgae culture.

1.7.6 Sustainable Yields

The maximum sustainable yield of a cultured microalgae is the maximum volume of culture that can be removed and replaced with algae free culture whilst maintaining the algae population. The culture is therefore kept at maximum productivity. The growth characteristics of microalgae cultures have been described comprehensively by Fogg (1975) for both batch and continuous culture systems. After an initial lag phase, microalgae cultures experience a period of exponential growth before reaching a plateau followed by decline. The maintenance of a culture in the exponential growth phase by a continuous input and output of either nutrients (chemostat) or culture (turbidostat) maximises yields. The nutritional value of the cultured cells is also maximised when they are in log growth phase (Brown et al., 1989). The purpose of the microalgae culture is to provide food for *Artemia. A* turbidostat culture with continuous removal of microalgae culture brine to an adjacent *Artemia* pond and the replacement of this brine with fresh culture is envisaged. The inputs and outputs will be regulated to ensure the microalgae remains in log growth phase and will need to be determined for the developed microalgae culture system.

1.8 Summary and Scope of Required Experimental Work

A review of the literature provided a great deal of important information directly relevent to the design of a large-scale cyst producing facility. The scope of required experimental work was also identified and is summarised below.

1.8.1 Microalgae Food Supply

The natural food for *Artemia* is brine microalgae. These would form the basis of a large-scale brine based feeding system but there is little information on practical techniques for mass

culturing these microalgae, particularly in open ponds, and there are very few reports of large-scale experiments conducted under semi-controlled conditions. The physiology of these algae is, however, well understood so fundamental experimentation will be unnecessary. The experimental work will concentrate on largely empirical, large-scale culture experiments specifically designed to develop practical techniques for the production of microalgae food in outdoor ponds. The production of microalgae food is, however, fundamental to the *Artemia* experimental work which forms the core of the thesis so is covered in the first of the experimental chapters (Chapter 3).

1.8.2 Growth response and Efficiencies of *Artemia*

There is a great deal of published information on the growth response and efficiencies of *Artemia* to a range of foods but there are many contradictions and the growth responses to the brine microalgae that are the natural food for *Artemia* have not been tested. The effect of intraspecific competition has also been poorly addressed and this is a key factor from from both an ecological and practical viewpoint because a rapidly reproducing population will quickly create conditions of intense competition.

1.8.3 Cyst Production

The reproductive biology of *Artemia* has been thoroughly researched but the switching mechanism from ovoviviparity to oviparity has not been identified. There are few reports on large-scale cyst production experimentation and the effect of intraspecific competition on cyst production has not been considered. Large-scale cyst producing experiments will form the core of the experimental work in the thesis which will be presented in Chapter 5.

1.8.4 Operational Facility

Published information on intensive *Artemia* production systems is limited to biomass production on a relatively small scale. The practical application of experimental findings into an operational system will involve a great deal of empirical experimentation.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Statistics

All statistical analysis in the following experimental chapters was undertaken with "Statgraphics version 6" (Copyright 1992, Manugistics Inc. 10987654321). Most analysis involved one and two way Analysis of Variance (ANOVA) techniques. The data was obtained from randomised and replicated experiments and homogeneity of variance was checked using Cochran's test and appropriate transformations applied to heterogenous or obviously non-normal data prior to analysis.

All salinities are expressed as brine density (g/mL) which is standard for saltfield and other workers dealing with hypersaline brine. All historical brine salinity records at Dampier Salt were measured and recorded as brine density (g/mL). A conversion table detailing chemical characteristics of seawater concentrates from seawater to well beyond salting point is appended (Baseggio, 1974, Appendix 1). This table includes conversions from brine density (g/mL) to salinity (ppt).

2.2 General Facilities

All equipment and facilities used in the project were supplied by Dampier Salt Limited who maintain a well equipped brine Research Laboratory. Detailed methods for individual experiments are included with the experimental reports in the following chapters but a general overview of equipment and facilities is included below.

2.2.1 Laboratory

Laboratory experiments were undertaken on benches in an air conditioned laboratory with a controlled and constant temperature of about 20^{o}C and illuminated with "Gro-lux" fluorescent tubes with a light intensity of about 1,500 lux on a daily cycle of 12 hours dark and 12 hours night. Laboratory equipment included an Environmental Chamber (for laboratory microalgal experiments); Nikon Research Microscope (for counting microalgae); Nikon Dissecting Microscope (for counting *Artemia* offspring and measuring animals) and; analytical instruments including Triac pH Meter; YSI Model 57 Dissolved O_2 Meter; Anton Paar DMA 46 four decimal point Brine Density Meter (this instrument works on the principle of resonance and automatically adjusts the brine temperature to 20^{o}C); and Licor Quantum Sensor (for monitoring light intensities). Phosphate

and iron analysis was undertaken colorimetrically with the aid of a Shimadzu UV/Visible Spectrophotometer using techniques described by Strickland and Parsons (1968). The laboratory was also equipped with a range of centrifuges and filtration equipment.

2.2.1 Outdoor Facilities

Most outdoor experiments were undertaken in 1,000 L fibreglass tanks (Plate 1) but 2,000 L and 5,000 L tanks were also available and used as indicated in subsequent chapters. A range of air blowers, compressors and pumps in combination with various Air Water Lift (AWL) and bubbling systems were used for aeration and circulating culture brines. The outdoor experiments were often undertaken adjacent to large test ponds of volume about 800 m^3 (Plate 2, Plate 3) which was used as a source of microalgal food for some experiments and which were ultimately used in pilot cyst production trials (Chapter 6). Overhead manifolds were constructed for controlled input of microalgae culture (food); fresh water to replace evaporative losses and; air supply. Tanks were equipped with various underflow and overflow screens as appropriate for the experiment being conducted.

2.2.3 Other Equipment

A range of nets, trays and drying ovens (40^{o}C for 24 hours) were used for collecting and drying cysts. Continuous monitoring of parameters such as Dissolved Oxygen was possible with the use of data loggers down loading onto a 486, DX2 computer.

2.3 General Methods

2.3.1 Microalgae Trials

All laboratory trials were conducted with brine collected from the salt field of brine density about 1.08 g/mL that was first micro-filtered through a GF/C filter paper and autoclaved at 120^{o}C. The brine was placed in labelled 250 mL conical flasks stoppered with cotton wool and these were placed at random in an environmental chamber. The microalgae used were *T.suecica* and *D.salina* which had previously been isolated from the Dampier brines and maintained as stock cultures in the

laboratory. Population density of microalgae cultures was monitored on a haemocytometer with cell numbers over a total of five specific squares, each encompassing 0.1 mm^3, counted on each side of the chamber. The total volume of these was 1 mm^3.

Outdoor culture brines were generally unsterilised, or sterilised with liquid sodium hypochlorite at a level >20 ppm chlorine. When brines were chlorinated it was found to be necessary to allow three days prior to inoculation with microalgae or the cultures would not grow. The nature of the inhibitory byproduct of the chlorination process is not known but chlorine itself became undetectable within 30 minutes in cultures aerated in full sunlight.

2.3.1 *A.franciscana* Experiments

All experimental animals were hatched from cysts collected directly from the Dampier saltfield. Techniques for measuring, weighing and counting experimental animals in the laboratory are fully detailed in Chapter 4. Measurement involved the temporary stranding of animals in the well of a cavity slide under a dissecting microscope equipped with a calibrated eyepiece graticule. They were then measured from the nauplius eye to the telson before being reflooded and transferred back to their beakers. Larger experimental animals were measured in the same way, with the brine slowly withdrawn as the animals swam forwards to ensure they were stranded in an extended position.

Techniques for accurately determining population size, reproductive output and feeding and harvesting strategies in both 1,000 L tank experiments and in the 800 m^3 trial ponds evolved during the course of the project. These were sometimes developed from first principles as there is a scarcity of published information on this type of experiment but techniques included in Sorgeloos et al., 1986 were often used a starting point. Full experimental details are included with the individual experiments in Chapters 4 and 5 but major innovations included the following:

2.3.1.1 Population Monitoring

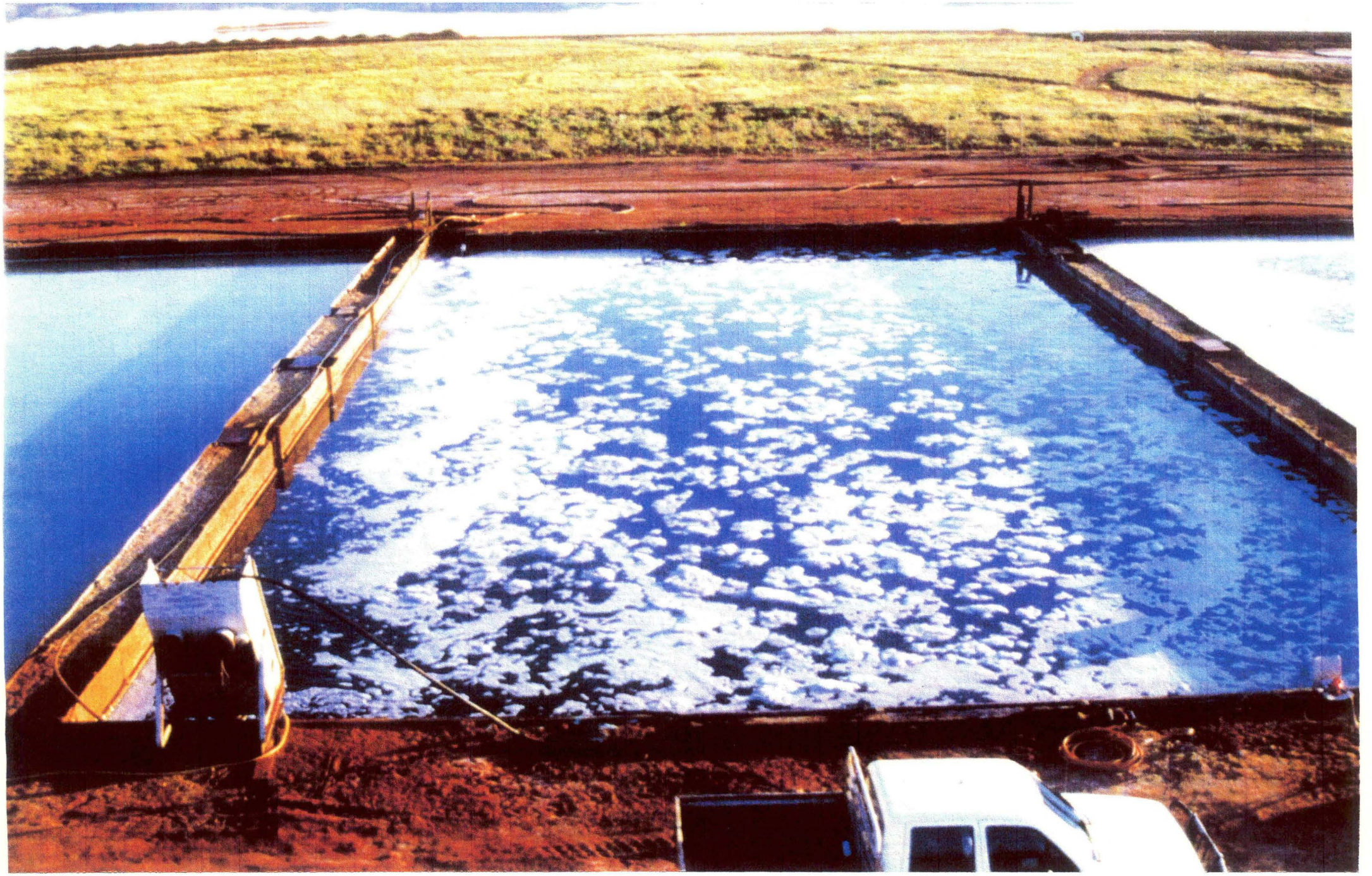
Populations of animals within 1,000 L tanks were determined by first homogenising the culture brines by vigorous stirring with an oar and taking a 1 L sample in a wide mouthed jar. The contents of this jar were poured through a 1 mm sieve and total numbers of both retained animals and fecund females (those with conspicuous ovisacs) were counted.

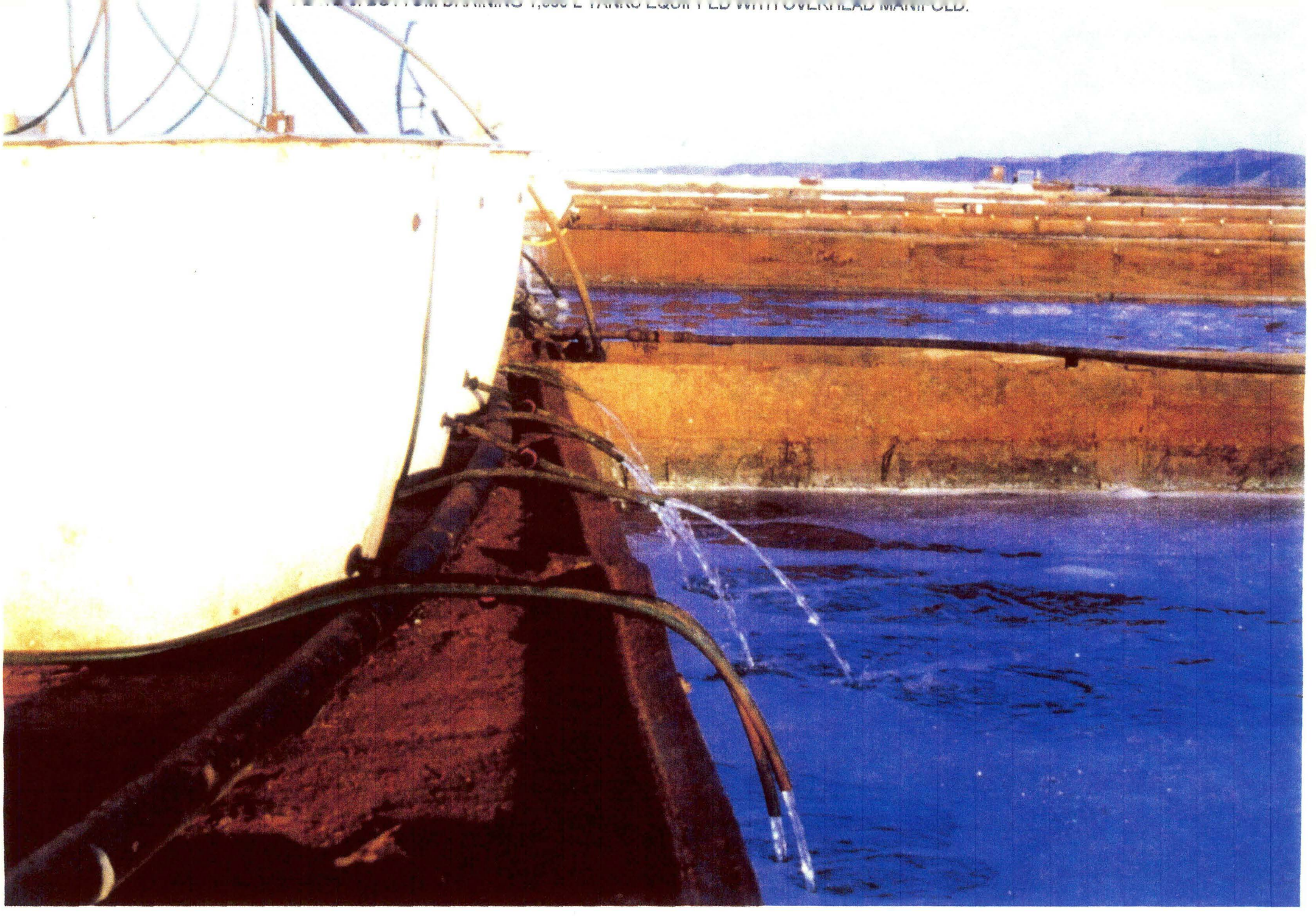
Captured animals were then washed back into the tank and the process repeated. This was done five times per tank with mixing between samples.

2.3.1.2 Reproductive Output

The technique developed to determine reproductive output accurately first involved determining the population of fecund females (see Section 3.3.1.1). Ten fecund females per culture vessel were captured using a glass tube and transferred to filtered brine in a beaker. Extreme care was taken to avoid any transfer of nauplii or cysts with the adult animals. The females were left for three days (this is the approximate reproductive time period determined from cyst producing experiments) to spawn. The adult females were then carefully removed with care taken not to remove any offspring. The contents of the beaker were then poured through a gridded filter paper and a count made of all spawned cysts and nauplii.

PLATE 1: 1,000 L FIBREGLASS TANKS USED FOR LARGE-SCALE OUTDOOR EXPERIMENTS.





CHAPTER 3. MICROALGAE MASS CULTURE

3.1 Introduction

Maintenance of a healthy phytoplankton population is considered one of the important keys for successful Artemia production in ponds (Tackaert and Sorgeloos, 1991). Tetraselmis suecica and Dunaliella spp. are the major genera of brine microalgae grazed by A.franciscana in the saltfield at Dampier. With a basic overall aim of developing a practical and reliable large-scale food supply for Artemia, four critical study areas were identified (Section 1.7). With the exception of overcoming practical problems in the mass culture of microalgae, the basic science of microalgae culture is well understood. The experimental approach in this Chapter, therefore, tended to be empirical.

3.1.1 Determination of Fertiliser Type and Level

In their review of microalgae for mariculture De Pauw and Persoone (1988) concluded that much more study was required into the dose-effect relationships of different inorganic fertilisers on specific types of microalgae, particularly those in marine environments. The use of commercially available inorganic fertilisers is an intermediate approach between the expensive defined media used for small-scale indoor culture of specific microalgae and, the cheap organic fertilisers traditionally used for semi-intensive microalgae production in the Middle-East and SE Asia for a range of aquaculture including Artemia culture (Vos et al., 1984).

3.1.2 Optimisation of Physico-Chemical Conditions

Physico-chemical conditions have to be optimised for maximum productivity and to sustain cultures for long periods because the unaccountable and frequent collapse of algae cultures is a major and ongoing problem in mariculture (De Pauw and Persoone, 1988). Intermittent injections of CO_2 were necessary to maintain *D.salina* cultures in a study by Olaizola et al. (1991). Salinity, degree of aeration and light intensity are three easily manipulated brine variables considered worthy of investigation.

3.1.3 Determination of Maximum Sustainable Yields

The upper limit for algal production appears to be in the range of 30 to 40 g dry weight m^2/day although the maximum recorded productivity of *D.salina* is 60 g dry weight/m^2/day (Borowitzka and Borowitzka, 1988). The 10 h doubling time achieved for Great Salt Lake *Dunaliella* (Van Auken and McNully, 1973) is a further indication of very high potential sustainable yields with this genus. Oswald (1988) used a formula; productivity (g/m^2/day) = 0.01 X depth (cm) X cell residence time (days), to predict theoretical yields of up to 36 short tons/acre/year achievable from a properly located and designed system, but concluded that such predictive equations are of limited value and that local in situ experiments are needed to obtain accurate productivity data.

3.1.4 Development of Remedies for Problems

All large-scale culture systems will be subject to a range of problems that need to be overcome for commercial success. Intensive cultures of micro-algae are subject to contamination and grazing by unwanted consumers including viruses, fungi, protozoans and zooplankton (De Pauw and Persoone, 1988). Protozoan contamination, which can devastate cultures in less than 24 h, despite the use of sterilisation and ultrafiltration techniques, is the most common problem (De Pauw and Persoone, 1988). In the case of a microalgae system adjacent to *Artemia* ponds, the control of *Artemia* contamination is another important consideration.

3.2 Optimising Culture Conditions for Brine Microalgae (Indoor Trials)

A number of complete soluble fertilisers are available commercially. One of these, "Thrive", was found to be effective in stimulating the growth of microalgae in brine of density 1.08 g/mL at Dampier Salt in 1983 at a level of 0.1 g/L (unpublished company records). Several experiments were undertaken to determine the minimum effective fertiliser inputs and to trial a range of commercially available soluble fertilisers.

Fertilisers are expensive so the minimum quantity of the cheapest effective fertiliser makes economic sense for large scale culture even though most species can tolerate high levels of nutrients (Kaplan et al., 1986).

3.2.1 METHODS

Details of general techniques are included in Chapter 2 (Section 2.3.1).

3.2.1.1 Experiment 3.2.1: Determination of Level of "Thrive" Fertiliser on Growth of T.suecica and Comparison of "Thrive" with the Direct Addition of Phosphate and Nitrate to the Brine.

A number of preliminary range-finding experiments were conducted which indicated a fertilisation level of 0.01 g/L was just as effective in promoting the growth of laboratory T.suecica as the 0.1 g/L level used historically. An experiment was designed to determine the optimum concentration of "Thrive" and to compare it with the addition of phosphate and nitrate added in isolation.

Two stock solutions were prepared:

1. 1 g "Thrive"/L of saltfield brine;
2. 8.75 g KNO_3 + 1.23 g KH_2PO_4 /L of saltfield brine (N:P ratio same as Thrive)

These were used to prepare four batches of culture brine:

1. 0.001 g Thrive/L
2. 0.01 g Thrive/L
3. 0.1 g Thrive/L
4. 0.01 g N+P (as nitrate and phosphate)/L

200 mL of each treated brine was placed in each of three labelled 250 mL conical flasks and inoculated with three drops of *T.suecica* culture from a Pasteur pipette before being placed in the environmental chamber which was set on 12 h light (1400 lux) and 12 h dark, a temperature of 30°C, and the culture shaker at 120 rpm. The initial and final pH and brine densities were determined and the population density of *T.suecica* monitored weekly for the final five weeks of the seven week trial.

3.2.1.2 Experiments 3.2.2 a-c: The Growth Response of T.suecica *and* D.salina *to Four Commercially Available Soluble Fertilisers in Brine of Density 1.07g/mL Collected From the Salt Field.*

A number of soluble fertilisers are available commercially (Table 3.2.1). Several experiments was undertaken in the laboratory to determine the growth response of both *T.suecica* and *D.salina* to four of the most commonly available fertilisers and to determine

which of these was the most suitable in terms of effectiveness, availability and price.

Table 3.2.1: Comparison of major macronutrients in the fertilisers as per manufacturers statement on container (full details in Appendix 5).

Fertiliser	Aquasol	Cropspur	Thrive	Zest
Phosphorous	4%	4%	4.5%	4%
Nitrogen	23%	24%	31%	23%
Potassium	18%	18%	9%	18%

Three trials (a-c) were conducted with identical methodology. The first using the Dampier strain of *T.suecica*; the second and third using the Dampier strain of *D.salina.*

The previously micro filtered and autoclaved brine of density 1.072 g/mL collected from the salt field was fertilised with each of the four fertilisers at levels of 0.01 and 0.1 g/L. 200 mL of each of these eight brines was then placed in two 250 mL conical flasks and the brines were inoculated with the appropriate strain of microalgae before being placed at random in the environmental chamber set at 25°C; 12 h light/dark cycling; light intensities at 1650 lux and; agitation at 1,200 rpm.

The 16 flasks used in the *T.suecica* trial (a) were inoculated with 1 mL of homogenous culture containing 48,000 cells/mL. The flasks were incubated for three weeks and the number of cells present in the flasks at the end of this period were counted on a haemocytometer.

The 16 flasks used in the first (b) of the two *D.salina* trials were inoculated with 1 mL of culture containing 560,000 cells/mL. The population of cells was monitored periodically from Day 4 until Day 18, at the end of the trial.

The repeat of the *D.salina* trial (c) ran for 13 days and with populations monitored on Day 6 and Day 13.

3.2.1.3 Experiment 3.2.3: Determination of the Optimum Level of Fertiliser for D.salina *in Laboratory Culture.*

Experiment 3.2.2 indicated that a fertilisation level of 0.1 g/L gave a much greater growth response than 0.01 g/L with *D.salina*. As these levels are an order of magnitude different it was necessary to run a trial at intermediate fertilisation levels.

Thrive fertiliser was added to flasks in triplicate for four treatments with identical methodology to Experiment 3.2.2 (above).

1. 0.02 g/L
2. 0.04 g/L
3. 0.06 g/L
4. 0.08 g/L

The 200 mL of fertilised brine in each of the twelve 250 mL conical flasks was inoculated with 0.5 mL of *D.salina* culture containing 1,010,000 cells/ mL. The trial was monitored for six days before problems with the environmental chamber caused the populations to collapse.

3.2.1.4 Experiments 3.2.4 (a-b): The effect of brine density on the growth of T.suecica *and* D.salina *in laboratory culture.*

Brine microalgae, including *Dunaliella spp.,* have complex ionic regulation systems enabling them to maintain cell hydration levels. These are essential for both the physical and chemical processes of microalgae in hypersaline brine (Richmond, 1986). *D.salina* has the highest salinity tolerance of all microalgae in the Dampier salt field. *T.suecica* dominates field brines of density less than 1.08 g/mL in Summer and *D.viridis* dominates the same brine density range during winter. Brines of density greater than 1.10 g/mL are dominated by *D.salina* throughout the year.

Laboratory brine density trials were undertaken to determine the optimum brine density to maximise productivity of both *T.suecica* and *D.salina. D.viridis* was not tested because it would not grow in the environmental chamber in the laboratory. It is probable that the 1,700 lux maximum light intensities in the environmental chamber were too low, because *D.viridis* grew well outdoors in identical culture media but with light intensities exceeding 7,000 lux at noon.

The *T.suecica* trials were undertaken prior to the brine fertilisation experiments and standard culture media was used. The *D.salina* trial was undertaken with brines fertilised with 0.1 g Thrive /L known at the time to support good growth.

3.2.1.4 a. T.suecica *Trials*

The growth rate of *T.suecica* recently isolated from the salt field (Dampier strain) was compared with the growth rate of laboratory trained *T.suecica* obtained from the CSIRO Marine Laboratories in Hobart in brine of a range of densities from seawater (1.025 g/mL)

to 1.075 g/mL. F2 media (Guillard and Ryther 1962) without silica was prepared using filtered brines collected from the salt field.

Aliquots of 150 mL were placed in 250 mL flasks, autoclaved at 120°C for 20 minutes and allowed to cool before being inoculated with 1 mL of the appropriate strain of *T.suecica.* The trial had two replicates with flasks placed at random in an environmental chamber set at 25°C, light intensity 1,500 lux, shaker on 1,200 rpm and light/dark cycling every 12 hours. The experiment ran for three weeks with algal populations monitored periodically during the last two weeks.

At the completion of the trial a repeat trial using saltfield *T.suecica* was undertaken in the brine density range 1.05 g/mL to 1.10 g/mL for a period of 21 days.

3.2.1.4 b. D.salina *Trial*

The *D.salina* trial was undertaken with brine of density 1.08 g/mL collected from the saltfield and filtered through GF/C filter papers and autoclaved. AR grade NaCl salt was added to some of this brine to produce brines of density 1.10 g/mL and 1.12 g/mL respectively. 150 mL of each of these brines was placed in each of three labelled 250 mL conical flasks before being inoculated with recently isolated *D.salina* culture and incubated at random in the environmental chamber (set as above). A fourth treatment using brine of density 1.08 g/mL fortified with Sodium Bicarbonate at a level recommended for *Dunaliella* in published media (Borowitzka, 1974) was also set up. This was done to determine whether carbon (not present in Thrive) supplements would be beneficial. The experiment ran for eight days.

3.2.2 Results

3.2.2.1 Experiment 3.2.1: Determination of Level of "Thrive" Fertiliser on Growth of T.suecica *and Comparison of "Thrive" with the Direct Addition of Phosphate and Nitrate to the Brine.*

The final pH values were significantly different between treatments but within a narrow pH band from 7.85 to 7.96 (Table 3.2.2). Final brine densities (g/mL) were not significantly different between treatments (Table 3.2.2). Brine pH reduced from an initial range of 8.02- 8.26 to 7.76-7.97 on Day 39. This decrease may have been caused by the brine density increase from the initial range of 1.0715- 1.0733 g/mL up to 1.0780-1.0860 g/mL on Day 39.

The population of *T.suecica* during the trial is shown in Table 3.2.3. The best productivity was achieved with "Thrive" concentrations of 0.01-0.1 g/L. These cultures significantly outperformed the 0.01 g (N+P) treatment which in turn had significantly higher productivity than the 0.001 g Thrive /L treatment.

Table 3.2.2 Final (Day 39) pH and density (g/mL) in Experiment 3.2.1 (Mean +- SE).

Thrive (g/L)	Final pH	Final Density (g/mL)	n
0.001	7.94 ± 0.13^{ab}	1.081 ± 0.0024	3
0.01	7.89 ± 0.003^{a}	1.0825 ± 0.0004	3
0.1	7.85 ± 0.05^{bc}	1.0810 ± 0.0006	3
0.01(N+P)	7.96 ± 0.003^{c}	1.0812 ± 0.0011	3
Probability	0.024	0.87	

For a single sampling time, means which share a common superscript are not significantly different (P>0.05)

Table 3.2.3: Two Way ANOVA table of *T.suecica* populations (cellsX10^3 /mL) over time in Experiment 3.2.1

Thrive (g/L)	Mean	SE	n	p
0.001	8.73^{a}	5.22	15	
0.01	56.53^{b}	5.22	15	0.0000
0.1	69.8^{b}	5.22	15	
0.01 (N+P)	34.9^{c}	5.22	15	
Time				
Day 16	33.25	5.84	12	
Day 20	29.7	5.84	12	0.0296
Day 25	49.4	5.84	12	
Day 31	49.5	5.84	12	
Day 39	50.5	5.84	12	

Means sharing a common superscript are not significantly different. There was no significant interaction (p>0.05).

3.2.2.2 Experiment 3.2.2: The Growth Response of T.suecica *and* D. salina *to Four Commercially Available Soluble Fertilisers in Brine of Density 1.07g/mL Collected From the Salt Field.*

A comparison of *T.suecica* population after three weeks culturing is shown in Table 3.2.4. Growth was greater with 0.1 g/L fertiliser than with 0.01 g/L to a highly significant degree ($p<0.01$) and the growth response with "Aquasol" was inferior to the other fertilisers. This experiment was confounded by the fact that much of the laboratory cultured *T.suecica* was growing on the sides of the culture vessels despite constant agitation.

The population of *D.salina* in the first trial after 13 days culturing is shown in Table 3.2.5. The 13 day data was chosen as a comparison with the repeat trial which only ran for 13 days and because there was no further population growth after this. There were highly significant differences in microalgae populations in both trials as a result of fertiliser level with superior performance at the 0.1 g/L level (Tables 3.2.5 and 3.2.6). Fertiliser type had no significant effect in either trial.

Table 3.2.4: Population of *T.suecica*$*10^3$/mL after three weeks (Two- way ANOVA). Mean+-SE(n) in Experiment 3.2.2a.

Fertiliser level(g/L)	Fertiliser Type				Mean
	Thrive	Zest	Aquasol	Cropspur	
0.01	8+-0.7(2)	6.5±1.06(2)	7.5±3.9(2)	11.5±0.35(2)	8.4±1.3(2)[a]
0.1	2.5+-1.06(2)	5±0.71(2)	6.5±4.9(2)	15.5±0.35(2)	7.4±2.0(2)[a]
Mean	5.25+-1.75(4)[a]	5.75±0.85(4)[a]	7.0±2.68(4)[a]	13.5±1.19(4)[b]	

Means that share a common superscript are not significantly different ($p>0.05$). There was no significant interaction.

Table 3.2.5: Population of *D.salina* $*10^3$ /mL after 13 days* in Experiment 3.2.2b. (First trial) Mean+-SE (n=2).

Fertiliser level(g/L)	Fertiliser Type				Mean
	Thrive	Zest	Aquasol	Cropspur	
0.01	1150±300	862.5±62.5	1075±87.5	1163±163	1063±65[a]
0.1	3906±812	4625±500	4587±488	3988±88	4277±198[b]
Mean	2831±1034[a]	2575±819[a]	2528±815[a]	2744±1105[a]	

Day 13 was chosen to allow comparison with second *D.salina* experiment (Table 3.2.5) which only ran for 13 days. Means that share a common superscript are not significantly different ($p>0.05$). There was no significant interaction. The mean biomass (dry wt.) was 0.2 mg/mL using conversion data presented in Appendix 4.

Table 3.2.6: Population of *D.salina* (cells $X10^3$ /mL) after 13 days in Experiment 3.2.2c (second trial). Mean+-SE(n=2)

Fertiliser level(g/L)	Fertiliser Type				Mean
	Thrive	Zest	Aquasol	Cropspur	
0.01	245+-35	285+-20	183.5+-34.5	155+-100	217.13+-23.7[a]
0.1	427.5+-38.9	555+-65	690+-0	3725+-27.5	536.3+-42.3[b]
Mean	436.8+-46.9[a]	313.8+-98.5[a]	336.3+-55.7[a]	420+-82.7[a]	

Means that share a common superscript are not significantly different ($p>0.05$). There was no significant interaction ($p>0.05$).

3.2.2.3 Experiment 3.2.3: Determination of the Optimum Level of Fertiliser for D.salina *in Experiment 3.2.3.*

Table 3.2.7: Comparison of *D.salina* populations (cells$X10^3$ /mL) after six days culturing in Experiment 3.2.3.

Treatment	Mean	SE	n	Prob.
1. 0.02 g/L	846.7	75.1	3	0.14
2. 0.04 g/L	973.3	96.1	3	
3. 0.06 g/L	625.3	111.3	3	
4. 0.08 g/L	741.7	91.7	3	

In the range 0.02-0.08 g/L fertiliser input ("Thrive") did not significantly affect *D.salina* population density ($p>0.05$).

3.2.2.4 Experiment 3.2.4a-c: The effect of brine density(g/mL) on the growth of T.suecica *and* D.salina *in laboratory culture.*

3.2.2.4 a. T.suecica *Trial 1 (3.2.4a)*

The population of the Dampier *T.suecica* was significantly smaller than the CSIRO strain (about 20%) and brine density had a highly significant overall effect (Table 3.2.8). There was a significant interaction between strain and brine density.

Table 3.2.8: Means Table and Two- Way ANOVA Probabilities for Population Density of Dampier *T.suecica* and *T.suecica* (CSIRO Culture Collection) At Three Brine Densities in F2 Media after 21 Days Culture (cells $X10^3$/mL).

Brine density /mL	Strain		Mean
	Dampier	CSIRO	
1.025	87±58(2)	752±58(2)	420±41(4)
1.05	290±58(2)	1041±58(2)	666±41(4)
1.075	171±58(2)	1196±58(2)	684±41(2)
Mean	183±33(6)	997±33(6)	

p values were; for strain (0.007); density (<0.001) and strainXdensity (0.047).

3.2.2.4 b. T.suecica *Trial 2 (3.2.4b)*

Inhibition by high salinities was apparent with Dampier *T.suecica* in the second trial with complete inhibition at a brine density of 1.10 g/mL (Table 3.2.9).

Table 3.2.9: Means Table and 1 way ANOVA probabilities for Dampier and CSIRO *T.suecica* After 21 Days in Brine of Density 1.05 to 1.10 g/mL in Repeat Experiment 3.2.4b. (cells $X10^3$/mL).

Treatment		Mean	SE	n
One.	1.05 g/m	207^a	5	2
Two.	1.075 g/	193.5^a	46.5	2
Three.	1.09 g/m	103.5^b	5	2
Four.	1.10 g/m	0^c	0	2
Probability		0.01		

Means sharing a common superscript are not significantly different (p>0 05).

The data failed Cochrans' homogeneity of variance test ($p<0.05$).

3.2.2.4 c. D.salina *Trial (Experiment 3.2.4c)*

D.salina grew well at all brine densities between 1.08 g/mL and 1.12 g/mL and the population was not significantly greater ($p>0.05$) in the treatment with added carbonate (Table 3.2.10). The eight day data were used because the populations of microalgae in several of the beakers had collapsed after ten days.

Table 3.2.10: Population of *D.salina*/mL*10^3 After Eight Days in Brine of Density 1.08 g/mL, 1.10 g/mL, 1.12 g/mL and 1.08 g/mL Fortified with Bicarbonate in Experiment 3.2.4c.

Treatment	Mean	SE	n
1. 1.08 g/mL	571.7	31.7	3
2. 1.10 g/mL	608.3	59.9	3
3. 1.12 g/mL	488.3	39.2	3
4. 1.08 g/mL+ carbonate	548.3	80.1	3
Probability	0.524		

3.2.3 Discussion

In Experiment 3.2.1 the greatest productivity of *T.suecica* was achieved in the 0.01 -0.1g/L "Thrive" treatments (Table 3.2.3). These treatments had significantly higher populations that the treatment with the equivalent amount of nitrate and phosphate indicating that "Thrive" contains beneficial nutrients in addition to Nitrogen and Phosphorus that are not being adequately provided by the brine (see Appendix 5). The level of 0.001 g "Thrive"/L was too low to give good growth.

In Experiment 3.2.2a, *T.suecica* numbers were low and variable but growth was significantly better at a fertilisation rate of 0.01g/L rather than 0.1g/L (Table 3.2.4).

D.salina grew much better at a fertilisation rate of 0.1 g/L than at 0.01 g/L in both *D.salina* trials (Tables 3.2.5 and 3.2.6) although cell numbers were higher by close to an order of magnitude after the same time period (13 days) in the first *D.salina* trial. All fertilisers gave a good growth response with no significant differences between the types of fertilisers.

It appears that the choice of fertiliser will be dictated by price and availability with no particular brand giving enhanced performance, particularly with *D.salina* culture.

In Experiment 3.2.3 the populations had not plateaued in the six day period at the end of the trial. The growth response at all fertilisation levels was, however, very good and not significantly different between treatments (Table 3.2.7).

It appears that a fertilisation level of 0.02 g/L of fertiliser is sufficient for good growth response in a laboratory environmental chamber at least over a six day period. In an applied situation in large scale culture the level of nutrients and population of microalgae could be monitored and additional fertiliser added on a periodic basis.

The first trial of Experiment 3.2.4 indicated a trend towards Dampier *T.suecica* growing better at lower brine density while the CSIRO *T.suecica* grew more prolifically at all brine densities compared to the Dampier strain (Table 3.2.8). The Dampier strain on the other hand had larger cells and a tendency to clump and become benthic. This made monitoring difficult even though the cultures were well agitated prior to counting.

The second *T.suecica* trial confirmed the pattern of growth inhibition by high brine densities (Table 3.2.9) and indicated virtually complete inhibition at a brine density of 1.10 g/mL. 1.10 g/mL is consistent with observations on the natural occurrence of *T.suecica* in the Dampier Salt Limited saltfield and is close to the ideal brine density for rearing cyst producing *A.franciscana* (see Chapter 5). This does not, however, preclude its use, because the brine density of the algae culture does not necessarily have to be the same as that for the *A.franciscana* culture although it would simplify the process.

The population of *D.salina* in a range of brine densities (g/mL), likely to be suitable for *A.franciscana* culture, was not significantly different after eight days (Table 3.2.10). Culture brine density (g/mL) tends to increase over time as a result of evaporative losses in the absence of freshwater. *D.salina* grows well in brine densities up to salt saturation (Borowitzka, 1974, Borowitzka et al., 1989) so population collapse as a result of salinity increase is unlikely.

3.2.4 Conclusion

The minimum concentration of soluble fertiliser required for good growth of *D.salina* in brine of density 1.07 g/mL in the laboratory was about 0.02 g/L with no increase in growth rate or final yield with the addition of higher concentrations after six days. The data for *T.suecica* was less clear but appears similar to that of *D.salina;*

T.suecica had lower productivity than *D.salina* on a cell number basis.

All four of the commercially available fertilisers tested were effective in promoting growth and were similar in their effects. The addition of nitrate and phosphate on their own gave inferior growth response compared to "Thrive". A fertilisation level of 0.02 g/L is much less than the 1 g/L concentration of macronutrients (excluding those abundant in brine) recommended by Borowitzka (1974) in standard culture media for Dunaliella. However, it depends on the anticipated culture period and whether the management strategy includes repeated additions of fertiliser for longer culture periods.

Aquasol was available in 25 kg bags from commercial suppliers near the saltfield and appeared suitable for use in large scale culture so was used in large scale trials (Chapter 6).

The population (cells/volume/day) of *Dunaliella spp.* in all trials was much greater than that of *T.suecica* (Tables 3.2.4 and 3.2.5). *T.suecica* also grows better at brine salinities below the optimum for *Artemia* cyst production at which competition from diatoms and other benthic organisms is much greater. Problems with predatory protozoans are also greater at lower brine density (discussed fully in Section 3.4). It appears, therefore, that *Dunaliella salina* are the preferable microalgae for culture and that a brine density of 1.10 g/mL is suitable for their growth.

3.3 Optimising Culture Conditions for Brine Microalgae (Outdoor Trials)

3.3.1 Introduction

Turbulence (Richmond, 1986b), brine salinity (Richmond, 1986b) and light intensity (Richmond, 1986a) are three easily manipulated factors known to affect the productivity of microalgae although more study into the effects of these variables is needed (De Pauw and Persoone, 1988). Culture mixing induces a fast movement of cells between the highly illuminated upper layer to the less luminated layers below and is one of the most basic requirements for high productivity in microalgal mass culture (Richmond, 1986).

The effect of aeration and shading on the growth and dominance of microalgae present in fertilised brine of density 1.055 g/mL collected from the salt field was assessed in a preliminary trial under winter conditions in June 1989 (Appendix 3). The brine was not sterilised

and contained a range of planktonic microalgae. *D.viridis* bloomed in aerated brine exposed to full sunlight but brines with all other combinations of shading and aeration remained clear.

The effects of brine density (g/mL), light intensity and degree of turbulence (via aeration) on the productivity (cells/volume/day) of *T.suecica, D.salina* and *D.viridis* in brine were tested in a series of experiments. It was also important to assess the value of adding additional fertiliser in large outdoor culture because of the cost of large inputs of fertilisers into large pond systems (Experiment 3.3.1). A further repeat of this experiment under summer conditions was also undertaken (Experiment 3.3.2).

3.3.2 Methods

Experiments involving shading and degree of agitation were undertaken in 1,000 L tanks outdoors.

Brine pH, and temperature were monitored in all experimental work on at least a weekly basis.

3.3.2.1 Experiment 3.3.1: The Effect of Fertilisation Strategy on D.viridis *in Outdoor Winter Culture.*

Four 1,000 L tanks (Plate 1) were filled with brine of density 1.065 g/mL collected directly from the saltfield. All tanks were under full sunlight and aerated conditions and fertilised with 0.01 g "Thrive" fertiliser/L. Two of the tanks received an additional 0.01 g/L on a weekly basis and two were not supplemented. The experiment was undertaken for three weeks.

3.3.2.2 Experiment 3.3.2: Effects of Shading and Aeration on Growth of Planktonic Microalgae in Fertilised Saltfield Brines in Outdoor Summer Conditions.

D.viridis grew spontaneously in fertilised salt field brine aerated in full sunlight in winter (Experiment 3.3.1). A repeat experiment under summer conditions was necessary because brine temperature, light intensity and day length are all greatly increased during summer. Previous unreported trials with fertilised brine undertaken in summer had always yielded *T.suecica* so a change in species dominance could also be a significant finding.

Twelve tanks were used to conduct an experiment encompassing four treatments in triplicate as follows, the experiment ran for 16 days. Brine density was initially 1.07 g/mL and brines were fertilised with 0.01 g/L "Thrive".

1. Shaded/aerated
2. Shaded/unaerated
3. Full sun/aerated
4. Full sun/unaerated

3.3.2.3 Experiment 3.3.3: Effect of Aeration Level on Growth of D.salina *in Fertilised Brine in Outdoor Summer (February) Culture.*

Experiments 3.3.1 and 3.3.2 demonstrated that vigorous aeration was essential to effectively mass culture *D.viridis* in field brines of density 1.07 g/mL fertilised with 0.01 g "Thrive"/L.

D.viridis would also grow spontaneously and become the dominant microalga in a 0.01 ha test pond receiving brine of density 1.07 g/mL directly from the saltfield. The brine was 1.5 m deep and fertilised with 0.01 g/L "Thrive". Aeration and agitation were achieved with an axial air blower delivering about 4,000 L of air/minute through perforated 32 mm black polypipe anchored to the floor of the pond. During 1990, for reasons not fully understood but suspected to be related to an expansion of the saltfield that occurred at this time (Burnard and Tyler 1993), there was a change in dominance from *D.viridis* to *D.salina* in the test pond. This reflected a similar change in dominance in the saltfield.

An experiment to determine the effect of aeration level on plankton growth in fertilised brine of density 1.07 g/mL collected directly from the saltfield was needed because the now dominant *D.salina* had been observed growing well without aeration in hypersaline brine both at the saltfield at Lake MacLeod (600 km south of the Dampier saltfield) and at a commercial Beta-Carotene facility adjacent to the Dampier saltfield.

The 0.01 ha plankton pond was drained and refilled with saltfield brine of density 1.07 g/mL. The brine was then fertilised with a 25 kg of "Aquasol" fertiliser to give an approximate concentration of 0.02 g/L and allowed to mix for 24 hours. Twelve, clean fibreglass tanks of 1,000 L capacity were then filled with fertilised brine pumped from the plankton pond. The tanks were aerated via standardised 25 mm stoppered PVC pipe with 3 mm diameter holes. The PVC pipe was connected via 13 mm garden hose to an overhead manifold fed from an axial air blower. Air flow to each

tank was controlled via individual ball valves to give the following aeration rates allocated at random:

1. No aeration
2. Gentle aeration (20 L/min)
3. Vigorous aeration (300 L/min).

Initial plankton, pH, brine density and phosphate levels were recorded for the brine used to fill the plankton pond. Phosphate and plankton levels were then determined for the pilot pond brine used to fill the tanks.

The experiment was monitored each week for three consecutive weeks with brine samples collected after mixing the brine in each tank with an oar. Brine pH, brine density (g/mL) and plankton were determined by the methods used in previous experiments.

A maximum/minimum thermometer was installed in a tank from each treatment on the first monitoring day and a diurnal trace of oxygen concentration was obtained for a single tank in order to determine times of maximum and minimum oxygen level. The diurnal range of oxygen was then measured at the determined times. The oxygen meter developed problems during the final week and reported data was from Week 2. A correction factor was applied to all meter readings on the basis of calibration tests (Appendix 2) which give results slightly lower than those predicted by Sherwood et al. (1992).

Numbers of planktonic protozoans (*F.salina*) were determined at the end of the trial by filtering 5 mL of culture brine through a GF/C filter paper and counting the clearly visible blue (unstained) dots on the paper.

The Experiment was repeated three times (a-c). The first was disrupted by rainfall and the second by *F.salina* contamination.

3.3.3 Results

3.3.3.1 Experiment 3.3.1: The Effect of Fertilisation Strategy on D.viridis *in Outdoor Winter Culture.*

Both brine pH and density increased greatly during the experiment (Table 3.3.1). There was a substantial reduction in levels of dissolved phosphate in tanks not receiving additional fertiliser and the temperature range within tanks was large. (Table 3.3.1). *D.viridis* spontaneously grew in all tanks but to a variable degree such that there was substantial variation between duplicates for the controls (no weekly fertilisation)(Table 3.3.2).

The addition of more fertiliser did not increase the population of *D.viridis*.

Table 3.3.1: Range of Physico-Chemical Parameters During Experiment 3.3.1

Treatment & Replicate	Temp.°C *	pH	Density (g/mL)	PO_4 (ppb) (range)
0.01 g/L Thrive once I.	14 - 27	8.12 - 8.19	1.070-1.096	682* - 31
0.01 g/L Thrive once ii.	10 - 24	8.11 - 8.96	1.069- 1.091	682 - 31
0.01 g/L Thrive weekly I.	12 - 27	8.09 - 8.68	1.069-1.088	682 - 1023
0.01 g/L Thrive weekly ii.	14 - 28	8.13 - 8.93	1.070-1.094	682 - 868

* Initial phosphate levels in all tanks calculated from the addition of 0.01g "Thrive"/L into brine with undetectable (<31 ppb) levels of PO_4^-.
** Brine temperatures were obtained using uncalibrated Max./Min. Thermometers had low accuracy (±1°C).

Table 3.3.2: Growth of *D.viridis* (cells/mL*10^3) During Experiment 3.3.1 (means)

Treatment	Day 1-5	Day 10-14	Day 17-20	Final Week Mean+-SE (n)
0.01 g/L Thrive once I.	250	3060	2588	2843±166(7)
0.01 g/L Thrive once ii.	150	770	1313	1321±113(7)
0.01 g/L Thrive weekly I.	25	938	1413	1314±121(7)
0.01 g/L Thrive weekly ii.	125	870	1413	1250±107(7)

3.3.2.2 Experiment 3.3.2: Effects of pH, Shading and Aeration on Growth of Planktonic Microalgae in Fertilised Saltfield Brines in Outdoor Summer Conditions.

The culture temperature range was about 10°C in all tanks and similar to the temperature range experienced in the saltfield brine concentrating ponds at the same time of year (Table 3.3.3). Brine pH rose greatly in unaerated tanks but not in aerated tanks with the exception of tanks in the pH adjusted treatments which returned to the normal level very quickly (Table 3.3.3). Initial

adjustment of pH had little effect on maximum pH (Table 3.3.3). Brine density (g/mL) increased in all tanks but particularly in the aerated tanks and there was a substantial decrease in the level of dissolved phosphate which became undetectable in all tanks exposed to full sunlight (Table 3.3.3).

D.viridis grew in all but one tank and *T.suecica* was present in many of the tanks irrespective of treatment after eight days (Table 3.3.4). There was a significant difference in the population of *D.viridis* between treatments after eight days, however, treatment effects on algal density, for either *T.suecica* or total combined microalgae populations were not significant (Table 3.3.4). The factor responsible for the difference in *D.viridis* populations was aeration (Table 3.3.5). The brine protozoan *F.salina* infested all but one tank by the end of the trial (Table 3.3.6) greatly reducing the populations of microalgae in the brine and preventing valid comparisons being made.

While *D.viridis* in shaded or unshaded pools did grow if aerated in summer, it did not grow well in shaded pools in winter (Appendix 3).

Table 3.3.3: Range of Physico-Chemical Parameters During Experiment 3.3.2

Treatment (replicate)	Temperature °C	pH	Density (g/mL)	PO_4
2.Shade -Air (1)	26-35	8.21-8.68	1.0687-1.0736	837-62
(2)	24-35	8.21-9.39	1.0687-1.0824	806-31
1.Shade +Air (1)	24-31	8.21-8.13	1.0687-1.0792	837-62
(2)	25-30	8.21-8.22	1.0687-1.0886	744-3
4.Sun -Air (1)	26-38	8.21-9.3	1.0687-1.0784	713-nd*
(2)	24-35	8.21-9.2	1.0687-1.0762	667-nd
3.Sun +Air (1)	23-32	8.21-8.43	1.0687-1.0876	558-nd
(2)	23-36	8.21-8.19	1.0687-1.0911	589-nd

nd = not detected (< 31 ppb)

Table 3.3.4: Comparison of treatment means of microalgae/mL*10^3 after eight days in Experiment 3.3.2

Treatment	*D.viridis*	*T.suecica*	Total	n
1.Shade -Air	4+-0.2^{ab}	2.8+-2.8	6.8+-3.0	2
2.Shade+Air	11+-0.8^{ac}	0.5+-0.1	11.5+-1.9	2
3.Sun-Air	2+-2^{b}	6.8+-6.8	8.8+-4.8	2
4.Sun+Air	15.3+-2.7^{c}	0+-0	15.3+-2.7	2
Probability	0.023	0.59	0.4	

Means that share a common superscript are not significantly different (p>0.05).

Table 3.3.5: Two- way ANOVA for eight day data *D.viridis* population (cellsX10^3/mL) in Experiment 3.3.2

Treatment	Mean	SE	n	Probability
A. Shading	7.5	1.6	4	0.63
	8.7	1.6	4	
B. Aeration	3.0	1.6	4	0.006
	13.0	1.6	4	

There was no significant interaction (p>0.05).

Table 3.3.6: Planktonic population on Day 16 (two replicates) in Experiment 3.3.2

Treatment (replicate)		*D.viridis*/mL*10^3	*T.suecica*/mL*10^3	*Fabrea*/L
1.Shade -Air	(1)	0.4	3.2	100
	(2)	2.25	2.6	1000
2.Shade +Air	(1)	1.2	0.4	2500
	(2)	2.4	1.6	5500
3.Sun -Air	(1)	2.6	0.2	300
	(2)	2.6	3.4	0
4.Sun +Air	(1)	15.4	0.4	500
	(2)	5	0.2	5400

Table 3.3.7: Comparison of light intensities and *D.viridis* growth in winter (June, Experiment 3.3.1) and summer (November, Experiment 3.3.2).

Treatment	Max. Light (lux)	*D.viridis*X10^3 /mL
1.Winter Sun	17500	>400
2.Winter shaded	2067	Nil
3.Summer Sun	23000	128
4.Summer Shaded	7000	160

Note: Winter figures are over a three week period (Appendix 3) and summer figures over an eight day period (Experiment 3.3.1)

3.3.3.3 Experiment 3.3.3a-c: Effect of Aeration Level on Growth of D.salina *in Fertilised Brine in Outdoor Summer (February) Culture.*

After 8 days culturing in an initial trial there were some indications that *D.salina* preferred aerated conditions and *T.suecica* calm conditions but data were too variable to be statistically meaningful (Table 3.3.8).

In the second trial there were highly significant differences between treatments in both brine pH and density g/mL (Table 3.3.9) with unaerated tanks having higher pH and lower density than their aerated counterparts.

After 12 days, the *D.salina* population was higher in the moderately aerated tanks but the difference was not significant and *T.suecica* was inhibited by aeration ($p<0.001$) (Table 3.3.10). This pattern was still apparent after 16 days but by this time the experiment was confounded by an infestation of *F.salina* in all tanks (Table 3.3.11).

Table 3.3.8: Population of microalgae (cellsX10^3/mL) after eight days in Experiment 3.3.3a (effect of aeration rate).

Treatment	*D.salina*/mL		*T.suecica*/mL		n
	Mean	SE	Mean	SE	
1. No Air	21.33	8.7	27.3	12.2	3
2. 20 L/min	88	44.7	1.3	1.3	3
3. 300 L/min	45	11	7	1	2*
Probability	0.34		0.14		

*One replicate of Treatment 3 was not fertilised (mistake) and data not included.

Table 3.3.9: Comparison of brine pH and density (g/mL) after 16 days culturing in Experiment 3.3.3b.

Aeration	pH		Density (g/mL)		n
	Mean	SE	Mean	SE	
1. No Air	9.42	0.03	1.079	0.002	3
2. 20 L/min	8.18	0.08	1.086	0.004	3
3. 300 L/min	8.07	0.007	1.095	0.001	3
Probability	0.0000		0.015		

Note: Initial PO_4 levels were about 500 ppb reducing to less than 5 ppb after 16 days in all tanks.

Table 3.3.10: Comparison of *D.salina* and *T.suecica* populations after 12 days in Experiment 3.3.3b.

Treatment	*D.salina*/mL*10^3		*T.suecica*/mL*10^3		n
	Mean	SE	Mean	SE	
1. No Air	4.6	1.1	16.7^a	1.1	3
2. 20 L/min	21.3	5.2	2.1^b	1.5	3
3. 300 L/min	15.4	5.9	0^b	0	3
Probability	0.1		0.0001		

Means that share a common superscript are not significantly different (p>0.05).

Table 3.3.11: Comparison of *D.salina*, *T.suecica* and *F.salina* populations after 16 days in Experiment 3.3.3b.

Aeration	*D.salina*/mL*10^3		*T.suecica*/mL*10^3		*F.salina*/L		n
	Mean	SE	Mean	SE	Mean	SE	
None	0	0	8.3	2.8	2400	808	3
20 L/min	9.3	5.9	2.3	2.3	16000	2705	3
300 L/min	4.3	1.2	0.3	0.3	14733	7194	3
Prob.	0.24		0.09		0.14		

When the experiment was conducted for a third time, the vigorously aerated tanks (Treatment 3) had significantly lower brine pH and significantly higher brine density (g/mL) than the other treatments with less aeration (Table 3.3.12). The non-aerated tanks in Treatment 1 had significantly higher minimum and maximum levels of dissolved Oxygen (Table 3.3.12).

Significant differences in populations of *D.salina*/mL were apparent after two weeks. Vigorously aerated tanks had higher population density than the tanks in the other treatments (Table 3.3.13). This pattern persisted into the third week despite an infestation of the brine protozoan *F.salina* which appeared to be inhibited by a high level of agitation (Table 3.3.13).

Table 3.3.12: Comparison of physico-chemical parameters during Experiment 3.3.3c.

Treat.	pH		Density(g/mL)		Min O_2 ppm		Max O_2 ppm		n
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
1.Air-	9.04^a	0.03	1.104^a	0.001	7.08^a	0.38	9.62^a	0.3	4
2.Air+	9.0^a	0.04	1.106^a	0.001	4.45^b	0.12	6.03^b	0.06	4
3.Air++	8.28^b	0.09	1.117^b	0.001	5.03^b	0	5.5^b	0	4
Prob.	0.0000		0.0000		0.0001		0.0000		

Means that share a common superscript are not significantly different (p>0.05).
Note: Initial pH was 8.03; density 1.0797 g/mL; PO_4^- 341 ppb; and brine temperature range during trial was 20 - 36°C.

Table 3.3.13: Comparison of *D.salina* population (cellsX10^3/mL) during 3rd repeat Experiment 3.3.3 and final *F.salina* population/L.

Treat.	*D.salina* W1		*D.salina* W2		*D.salina.* W3		*F.s*alina		n
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
1.No air	383[a]	55	95[a]	23	47[a]	25	19050[a]	1808	4
2.Air+	463[a]	6	187[a]	81	58[a]	28	22550[a]	1842	4
3.Air++	482[a]	40	1033[b]	250	1047[b]	296	4125[b]	1855	4
Prob.	0.22		0.0032		0.0037		0.0001		

Means that share a common superscript are not significantly different (p>0.05).

General Comment: Tanks with vigorous aeration had green coloured brine and little benthic growth. Tanks with low aeration and no aeration had clear brine and substantial amounts of benthic cyanobacteria, some of which was floating, by the end of the trial.

3.3.4 Discussion

The experimental work in section 3.3 was undertaken to develop practical techniques to optimise culture conditions for the successful mass culture of microalgae in brine in large outdoor ponds. A preliminary experiment on the effect of aeration and shading on the growth of microalgae in outdoor culture (Appendix 3) demonstrated good growth of *D.viridis* in aerated brine in full sunlight (17,500 lux at noon) while shaded tanks (2,000 lux at noon) grew nothing and tanks in full sun without aeration had some benthic growth (assorted cyanobacteria and diatoms). That trial also demonstrated that a once-off addition of "Thrive" fertiliser at a rate of 0.01 g/L was sufficient to support a high population ($4*10^6$ cells/mL) of *D.viridis* for the three week trial despite most of the phosphorous being utilised. Little reactive phosphate (<16 ppb) remained in the brine compared to about 500 ppb in shaded tanks without algae. The minimum concentration of fertiliser for maximum standing crop was determined as 0.02 g/L in the experimental undertaken in Section 3.2. The ability to mass culture microalgae without shading has significant economic benefits as does the high yield achieved with only 0.01 g/L of fertiliser. It was important to confirm these unreplicated initial findings (Experiments 3.3.1 and 3.3.2) and to conduct an

experiment to determine the minimum degree of aeration needed for good growth (Experiment 3.3.3).

Experiment 3.3.1 confirmed that good growth of *D.viridis* could be achieved in fertilised (0.01 g/L) brine aerated in full sunlight (Table 3.3.2). The *D.viridis* populations in tanks containing brine fertilised with 0.01 g/L thrive were similar to tanks receiving additional thrive (0.01 g/L/week) but most of the reactive phosphate had been utilised (<31 ppb remaining, Table 3.3.1). It appears that 0.01 g "Thrive"/L is close to the minimum level required to maximise the population of *D.viridis* and that it is efficiently recycled.

The large difference in *D.viridis* populations between the control replicates is difficult to account for. The difference in brine pH between these replicates was also large with the tank supporting most algae having a pH of 8.19 and it's poorer performing counterpart having a final brine pH of 8.96 (Table 3.3.2). The pH rise was possibly caused by the removal of CO_2 by *D.viridis* and perhaps the better performing culture had better aeration. In an unreported experiment pH was adjusted downwards with HCl. The pH quickly rose to levels very similar to those of the unadjusted brines experiencing the same conditions of full sunlight and aeration indicating that direct pH adjustment of brines is difficult without the use of buffers or regular inputs of acid.

Experiment 3.3.2 was undertaken to determine the effect of shading and aeration on the growth of *D.viridis* under summer conditions with maximum light intensities of 23,000 lux and was similar in design to the appended preliminary experiment (Appendix 3). The 16 day population data for microalgae was confounded by an infestation of predatory protozoans, *F.salina* necessitating the use of eight day data in statistical analysis (Tables 3.3.5 and 3.3.6). These data indicated that only *D.viridis* was significantly affected by the treatments. Growth of *D.viridis* was significantly enhanced by aeration consistent with the winter trials (Appendix 3 and Experiment 3.3.1) but unlike the winter trials good growth was achieved in both the full sun and the shaded conditions which had greatly increased peak light intensities (Table 3.3.7, 7,000 lux cf 2,000 lux in the winter trials). This indicates that *D.viridis* requires a minimum light intensity of 2,000 to 7,000 lux for at least part of the day, not withstanding self-shading and the short time duration of the highest intensities, and can tolerate at least 23,000 lux maximum light intensities. Photosynthetic efficiency increases if high intensity radiant flux (e.g. 2,500$\mu E/m^2$ /sec) is interspersed with short intervals of darkness compared to continuous light (Richmond and Becker, 1986) and this is the situation for individual cells in turbulent and relatively deep (1.5 m) outdoor culture tanks.

F.salina which infested the brines in the final week (Table 3.3.6) is a potential major problem in the mass culture of microalgae in brine (see Section 3.4).

An initial brine fertilisation rate of 0.01 g/L appears adequate to sustain good growth of microalgae but in full sun conditions the level of dissolved reactive phosphate had reduced to undetectable levels by the end of the trial.

In Experiment 3.3.3, the trend of growth of microalgae in the first trial (Experiment 3.3.3a), prior to disruption due to rain, was similar to that in the second trial (Experiment 3.3.3b) with *T.suecica* dominating unaerated brine and *Dunaliella spp.* dominating aerated brine (Table 3.3.8). The *Dunaliella* species was *D.salina* in contrast to previous experiments dominated by *D.viridis*. *D.salina* also dominated in the third trial (Experiment 3.3.3c). In the second trial (Experiment 3.3.3b) *T.suecica* dominated unaerated brine and *D.salina* grew best in moderately aerated brine (Table 3.3.10). At the end of the trial, infestations of the predatory protozoan *F.salina* in all tanks confounded the final results by consuming a proportion of the microalgae (Table 3.3.11). The population of *F.salina* was greatest in moderately aerated tanks.

Turbulence as a result of aeration evens nutritional and gaseous gradients and evenly distributes solar radiation to the cells (Richmond and Becker, 1986). Aeration also provides gases such as O_2 and CO_2 to the media, increases evaporation and helps purge the system of supersaturated gases. There were highly significant differences in both brine pH and density between treatments (Table 3.3.9). Brine pH was highest in unaerated tanks presumably because the lack of aeration prevented the input of significant amounts of CO_2 to replace the CO_2 being consumed by the microalgae. Brine density (g/mL) on the other hand was highest in vigorously aerated tanks presumably due to increased evaporation rates.

In Experiment 3.3.3c, there were significant differences in plankton densities between treatments after three days (Table 3.3.12). After ten days there was a significant difference between treatments with the vigorously aerated tanks having much greater levels of planktonic *D.salina* (Table 3.3.13). The tanks with low aeration and with no aeration had clear brine and conspicuous benthic growths dominated by filamentous cyanobacteria in the genera *Oscillatoria* and *Spirulina*.

The diurnal range of dissolved Oxygen was obtained by taking readings at 0600 and 1800 hours. The diurnal range was very small in vigorously aerated tanks in Experiment 3.3.3c compared to the other treatments and the non-aerated tanks had

significantly higher maximum and minimum Oxygen levels (Table 3.3.13). It is possible that super-saturation with photosynthetically produced oxygen was occurring in the lowly aerated tanks and that this was prevented by vigorous aeration. It is also possible that the large oxygen bubbles within the benthic communities in the lowly aerated tanks only slowly diffused into the water column to keep the oxygen levels elevated for some time.

The pH became greatly elevated in tanks without vigorous aeration (Table 3.3.12). The reasons for this are unclear but may be in part due to the ability of cyanobacteria to utilise bicarbonate as a carbon source (Kaplan et al., 1986). The relative concentrations of the inorganic carbon species determine the pH and in turn are determined by the pH (Kaplan et al., 1986). The removal of bicarbonate and CO_2 from brine will elevate the pH. Benthic cyanobacteria were abundant in tanks with little or no aeration. These tanks were receiving less atmospheric CO_2 than the vigorously aerated tanks but at the same time solubility of CO_2 will increase as pH increases. The removal of CO_2 from the brine increases the pH and causes CO_3^{2-} becomes the major inorganic carbon species. This may well have given the Cyanobacteria an advantage over the *D.salina* which initially grew well without aeration. Richmond (1986) reports that in mixed cultures of green algae (*Chlorella*) and Cyanobacteria (*Spirulina*), the cyanobacteria will always dominate as the pH rises. The addition of CO_2 lowers the pH and causes a dramatic reversal in dominance. Olaizola and Duerr (1992) found that periodic inputs of gaseous CO_2 were necessary to sustain outdoor cultures of *Tetraselmis chuii* for long periods.

It appears likely that the importance of aeration to the *Dunaliella spp.* cultures is in the provision of CO_2 stabilisation of pH. Benefits from turbulence appear to be secondary. *D.salina* grows well in unshaken flasks in the laboratory and often blooms in still ponds within the saltfield. Table 3.3.13 shows clearly that the effects of aeration only really become apparent after about two weeks of culture and that *D.salina* initially grows well without aeration. The experimental work described above was undertaken with normal air. It is possible that elevation of CO_2 levels in the air stream would have reduced the minimum aeration level necessary to provide the microalgae with their required CO_2 and to prevent pH increases.

The predatory brine protozoan *F.salina* was present in all tanks by the end of the trial. It did, however, appear to be inhibited to some extent in the vigorously aerated tanks (Table 3.3.13) and may not like turbulence. The apparent contradiction to this is the large numbers of *F.salina* in aerated tanks in Experiment 3.3.3b (Table

3.3.11) where there is a close relationship with *D.salina* numbers i.e. food supply.

3.4 Maximum Sustainable Yields

3.4.1 Introduction

The upper limit for algal production appears to be in the range of 30 to 40 g dry weight m2/day (Borowitzka and Borowitzka, 1988). The maximum recorded productivity of D.salina at 60 g Dry weight/m2/day (Borowitzka and Borowitzka, 1988) is higher than for most algae and the ten hour doubling time achieved for Great Salt Lake Dunaliella (Van Auken et al. 1973) is a further indication of very high potential sustainable yields. Hard data on achieved microalgae production in large scale culture is difficult to find. Oswald (1988) uses a formula (productivity (g/m2/day)=0.01*depth (cm)*cell residence time (days) to predict theoretical yields of up to 36 short tons/acre/year achievable from a properly located and designed system but concludes that such predictive equations are of limited value and that local in situ experiments are needed to obtain accurate productivity data.

The growth responses over time of populations of *D.salina*, *D.viridis* and *T.suecica* in the experiments described in Sections 3.1 and 3.2 give some indication of sustainable yields from a population perspective under a range of conditions. The sustainable yield of laboratory cultured *D.salina* (Section 3.1, Experiment 3.2.2) was as high as 25%, equivalent to (500,000 cells per mL per day) but was much less in outdoor cultures which were influenced by infestations of *F.salina* and competition with benthic algae.

The following experiments were designed to determine the maximum sustainable yield of *Dunaliella spp.* in mass culture.

3.4.2 Methods

3.4.2.1 Experiment 3.4.1: Maximum Sustainable Yields of D.viridis *in Outdoor Culture.*

The number of A.franciscana that can be supported by a particular volume of D.viridis culture will be dependent on the productivity of the algae. The following trial was conducted in order to determine the maximum sustainable yield of D.viridis in outdoor culture.

Six 1,000 L tanks were filled with brine of density 1.078 g/mL fertilised with "Thrive" fertiliser at a rate of 0.01g/L and aerated in full sunlight. The population of D.viridis was monitored in all tanks until no further population increase was apparent. Three treatments in duplicate were then applied to the tanks at random using culture brine identical to that used to establish the cultures. The treatments were as follows:

5% daily dilution.

10% daily dilution.

20% daily dilution.

The tanks were diluted with fresh brine (identical to that used to establish the cultures) provided by a gravity flow from an adjacent storage tank containing fertilised field brine housed in the dark to prevent growth of microalgae. A submersible poly-pump was used to remove brine on subsequent days prior to refill.

Plankton population densities and the range of brine temperatures were recorded daily by the methods outlined in previous experiments. Brine pH, density (g/mL) and level of dissolved reactive phosphate were monitored weekly.

3.4.2.2 Experiment 3.4.2: Effect of Brine Turn-Over on Populations of D.salina *in Saltfield Brine of Density 1.08 g/mL.*

Experiment 3.4.1 was confounded by *A.franciscana* contamination in all tanks. A repeat experiment was designed to determine the effect of brine turn-over rates of up to 40% on the growth of both microalgae and the brine protozoan *F.salina* that intermittently infests the brine.

The methods are basically as outlined in Experiment 3.4.1 but with four treatments in duplicate as follows.

5% daily turnover.

10% daily turnover.

20% daily turnover.

40% daily turnover.

Turnover commenced after Day 10 when the microalgae had become well established in all the tanks. Populations of *D.salina* and *F.salina* were monitored daily. Brine pH, density (g/mL) and level of reactive phosphate were monitored weekly.

3.4.3 Results

3.4.3.1 Experiment 3.4.1: Maximum Sustainable Yields of D.viridis *in Outdoor Culture.*

The ranges of physico-chemical parameters were similar between treatments (Table 3.4.1) and there was no significant difference between treatments in the population of *D.viridis* at the beginning of the trial or after one week (Table 3.4.2).

Table 3.4.1: Range of Physico-Chemical Parameters During Experiment 3.4.1.

Treatment	Temperature^{o}C	pH	Density (g/mL)	PO_4-ppb
5% Turnover	13-28	8.15-7.99	1.0774-1.0831	31-9.1
	15-27	8.09-8.18	1.0774-1.0830	31-9.3
10% Turnover	15-27	7.92-7.96	1.0784-1.0805	31-9.3
	16-27	8.12-8.15	1.0776-1.0806	31-9.3
20% Turnover	16-27	8.08-8.27	1.0778-1.0787	31-20
	16-27	8.29-8.57	1.0753-1.0783	31-17

Table 3.4.2: *D.viridis* Population/mL, Initially and After One Week.

Treatment (daily turnover)	Initial		1 Week		
	Mean	SE	Mean	SE	n
5%	2725	175	1700	550	2
10%	2700	200	2275	225	2
20%	2475	25	1325	175	2
Probability	0.53		0.31		

3.4.3.2 Experiment 3.4.2: Effect of Brine Turn-Over on Populations of D.salina *in Field Brine of Density 1.08 g/mL.*

There was a significant difference in final brine densities (g/mL) with tanks experiencing the highest brine turnover having lower densities (g/mL) but no significant difference in final pH (P>0.05)(Table 3.4.3).

The plots of microalgae populations (primarily *D.salina* but including some *T.suecica*) in Figure 3.4.1 and *F.salina* populations in Figure 3.4.2 clearly show the collapse of the microalgae populations after 17 days coinciding with a dramatic increase in *F.salina* population.

There were significant differences in *D.salina* populations over time ($p<0.001$) and between treatments ($p=0.095$) from Day 6 to Day 17 with a clear pattern of population increase over time and population reduction with increasing daily brine turnover (Table 3.4.4).

The population of *F.salina* on Day 17, prior to the collapse of the *D.salina* populations, was not significantly different between treatments but a highly significant difference between treatments was apparent on Day 24 with much higher *F.salina* populations in tanks with low turnover (Table 3.4.5).

The mean productivity of *D.salina* (using the 6.5×10^{-11}g/cell figure from Appendix 4) in terms of mean dry weight (g) removed/m^3/day is shown in Table 3.4.6 and shows a steady increase in productivity with increasing daily brine turnover.

Table 3.4.3: Brine Density (g/mL) and pH after 24 Days in Experiment 3.4.2.

Treatment (daily turnover)	pH		Density (g/mL)		n
	Mean	SE	Mean	SE	
5%	8.09	0.13	1.0926^{a}	0.0004	2
10%	8.1	0.06	1.0887^{ab}	0.0015	2
20%	8.16	0.03	1.0849^{bc}	0.0013	2
40%	8.17	0.05	1.0814^{c}	0.0002	2
Probability	0.86		0.0056		

Means sharing a common superscript are not significantly different ($p>0.05$).
Note: Temperature range was 25-33°C and final level of dissolved PO_4- was about 2,000 ppb in all tanks.

Table 3.4.4: Means Table of *D.salina* Population(cellsX10^3/mL) between Day 6 and Day 17 in Experiment 3.4.2. (Data for each time period averaged across treatment levels, data for treatment levels averaged across time).

FACTOR	Mean	SE	n
A: TIME			
DAY 6	7^{a}	4.9	8
DAY 10	8^{a}	4.9	8
DAY 11	7.5^{a}	4.9	8
DAY 12	9^{a}	4.9	8
DAY 13	9.9^{ab}	4.9	8
DAY 14	14.6^{ab}	4.9	8
DAY 15	23.1^{b}	4.9	8
DAY 16	43.8^{c}	4.9	8
DAY 17	57^{c}	4.9	8
B: TREATMENT			
5% d turnover	28.7^{x}	3.3	18
10% d turnover	19.8^{xy}	3.3	18
20% d turnover	16.9^{y}	3.3	18
40% d turnover	14.6^{y}	3.3	18

Means sharing a common superscript are not significantly different (p>0.05). SE values are based on mean square error. The interaction was not significant (p>0.05).

Table 3.4.5: Comparison of Treatment Means for Day 17 and Day 24 *F.salina* population/mL in Experiment 3.4.2.

Treatment (daily turnover)	Day 17		Day 24		n
	Mean	SE	Mean	SE	
5%	16	4	36.5^{a}	1.5	2
10%	9	2	17.6^{b}	2.4	2
20%	11.5	3.5	13.5^{bc}	4.5	2
40%	4	1	4.7^{c}	0.3	2
Probability	0.16		0.0046		

Means sharing a common superscript are not significantly different (p>0.05).

Table 3.4.6: Mean daily dry weight biomass (g) of *D.salina* removed from 1 m^3 culture vessel at four daily brine turnover rates up to Day 17 in Experiment 3.4.2.

Daily Turnover Rate	Mean Dry Weight (G) Removed/Day From 1,000 L Tank
5%	0.09
10%	0.13
20%	0.22
40%	0.38

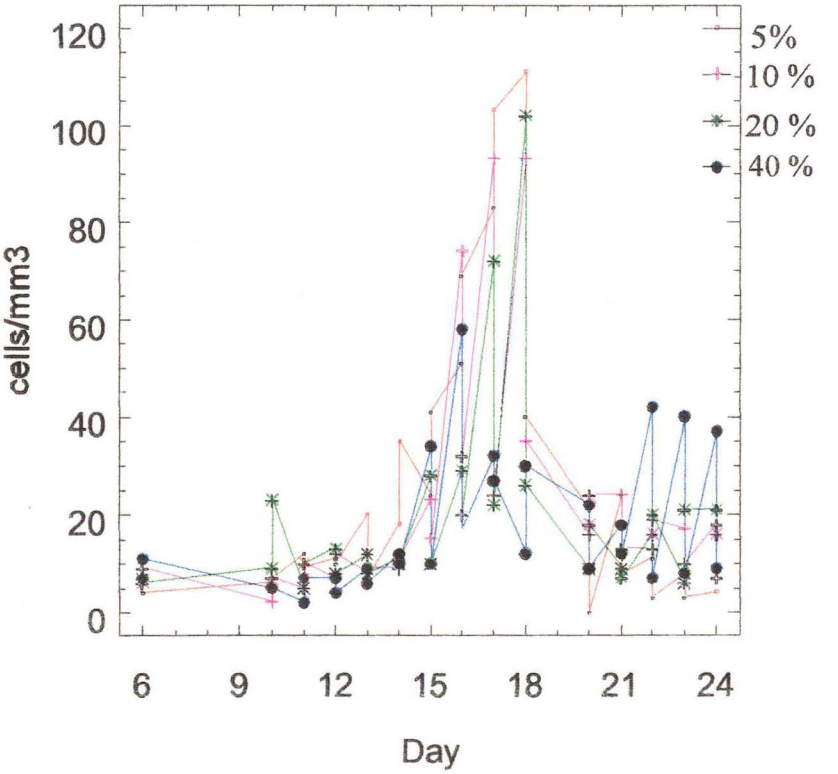


Figure 3.4.1. Plot of *D.salina* populations at four daily turnover rates over 24 days in Experiment 3.4.2.

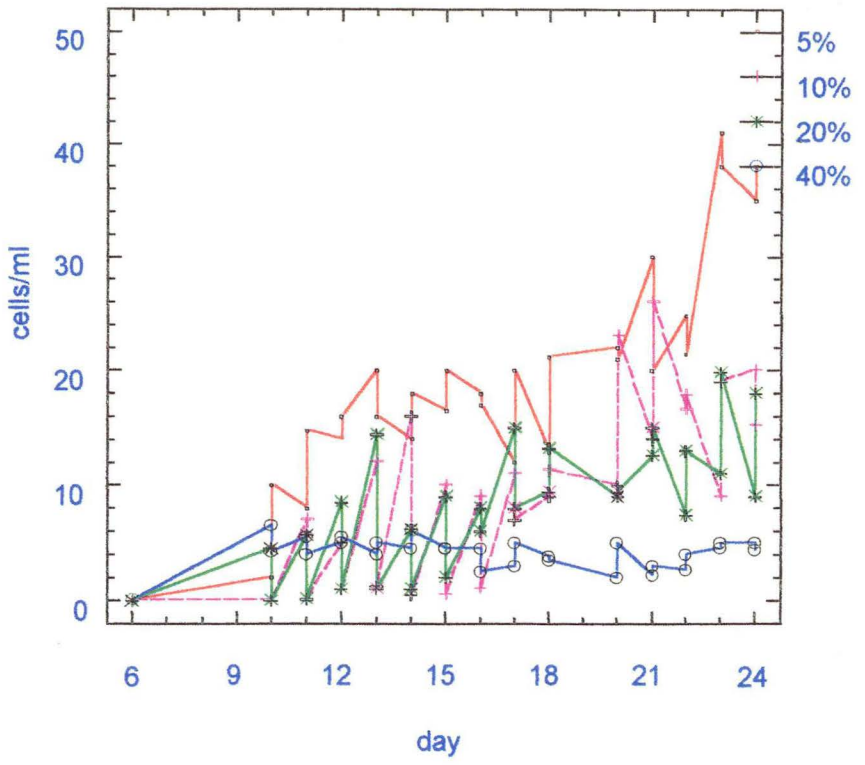


Figure 3.4.2. Population of *F.salina*/mL at four daily turnover rates over 24 days in Experiment 3.4.2.

3.4.4 Discussion

Experiment 3.4.1 was abandoned after one week due to infestations of *A.franciscana* in all tanks and although there was no significant difference in the population of *D.viridis* between treatments (Table 3.4.2), the experiment was inconclusive. It appears that the major problem in sustaining populations of *D.viridis* will be control of predators such as *A.franciscana* and *F.salina* which affected many of the experiments. These problems are addressed in Section 3.5.

In Experiment 3.4.2 microalgae grew at all turnover levels but growth was most rapid at the lowest daily turnover rate of 5% (Figure 3.4.1). *D.salina* populations were at their highest prior to the dramatic population collapse which preceded the rapid expansion in the population density of *F.salina* (Figures 3.4.1 and 3.4.2). The slow but definite increase in *D.salina* population at a daily turnover of 40% up to Day 17 indicates that it is possible to sustain cultures at this level of dilution (Figure 3.4.1, Table 3.4.4) provided that predators such as

F.salina can be controlled. There was a highly significant difference in the populations of *F.salina*/mL between treatments on Day 24 with highest populations in tanks with the lowest daily turnover of brine (Figure 3.4.1, Table 3.4.5) indicating some inhibition of *F.salina* population due to dilution from high daily brine turnover.

Table 3.4.6 shows a pattern of increasing daily dry weight (g) removal of *D.salina* with increasing brine turnover indicating that lower standing crops are more than compensated for by increased productivity at daily brine turnover rates of 40% compared to lower rates. This is because the effect of water exchange rate on unialgal density is non-linear. Specifically the average *D.salina* densities in the from Day 6-17 in Experiment 3.4.2 (Table 3.4.4) for each turnover rate are consistent with the pattern obtained by Allan and Maguire (1993) in model penaeid prawn farming ponds; the effect of water exchange on algal density was most evident in the range 5-10% turnover/day rather than at higher exchange rates (20-40%)

3.5 Control of Artemia *and* Fabrea *Contamination of Algal Cultures*

3.5.1 Introduction

Intensive cultures of micro-algae are subject to contamination and grazing by unwanted consumers including viruses, fungi, protozoans and zooplankton (De Pauw and Persoone 1988). Protozoan contamination, which can devastate cultures in less than 24 hours, despite the use of sterilisation and ultrafiltration techniques, is the most common problem (De Pauw and Persoone 1988). The dominant protozoan in the saltfield brine was *F.salina*. In the case of a microalgae system adjacent to *A.franciscana* ponds, the control of *A.franciscana* contamination is another important consideration. A third consideration is the control of undesired competitor algae.

Problems with protozoan infestation, *A.franciscana* contamination, and dominance of undesired microalgae species such as unicellular cyanobacteria were encountered in the course of experimentation and in pilot trials in large ponds. This led to the trialing and development of various control methods using a combination of laboratory test work and trials in large scale culture ponds (see Chapter 6). Much of this work was on an empirical rather than a detailed experimental basis and hence Section 3.5 is largely descriptive.

3.5.2 *Artemia* Infection

Laboratory trials (unreported) showed that quiescent *A.franciscana* cysts would hatch if placed in brine of density 1.08g/mL as indicated in other studies (Sorgeloos et al., 1986). Cysts are invariably present on the walls and floor of constructed ponds near the saltfield which has supported populations of *A.franciscana* for about 20 years. They can also enter the pond with wind blown foam from areas containing *A.franciscana,* and on birds. It is important, therefore, to ensure brine densities are maintained >1.08g/mL and for a margin of safety >1.09g/mL to prevent these cysts from hatching. The ability of *A.franciscana* to reproduce rapidly ensures that any contamination will quickly result in a clear plankton pond.

Saltfield brine used to flood large plankton ponds invariably contains some A.franciscana nauplii. It is important that these are killed or removed prior to the flooding of the pond. Preliminary bioassays conducted in the laboratory showed that chlorination at 10 ppm killed all A.franciscana. Another promising method was screening (150 mm) to remove the soft bodied nauplii, which are approximately spherical with a minimum length about 440 mm, and cysts about to hatch (about 200 mm). Despite all precautions, test ponds inevitably became contaminated with A.franciscana. The best method to minimise the impact of A.franciscana for as long as possible was to ensure the brine was initially free of them and then to continuously screen the brine to remove any contaminating animals. The use of an impellor driven "Aere- O_2" unit to aerate the plankton pond was found to be helpful because it tended to destroy the soft bodied A.franciscana.

3.5.2.1 Trialed Screening Methods

A.franciscana nauplii take about two weeks to reach sexual maturity, depending on food supply (Chapter 4). Continuous screening from Day 1 greatly inhibits the population of *A.franciscana* and enables control of *A.franciscana* numbers for up to several months. The following methods were trialed to screen the *A.franciscana* from the brine column.

3.5.2.1.1 SCREENING SOCKS OPERATED BY AIR WATER LIFT SYSTEM

Socks of mesh size 300 mm and dimensions 50 mm X 300 mm were tied to the outlets of a bank of Air Water Lift units and effectively removed *A.franciscana* from the water column. The degree of effectiveness was, as expected, dependent on the number of units, where they were placed, the degree of mixing in the pond, and the flow rate through each unit. In a large pond this method was found to be impractical. Large volumes of air and 40 Air Water Lift units effectively removed large quantities of *A.franciscana*

in the 0.1 ha pond but many avoided capture and eventually cleared the microalgae from the water column. The problem was exacerbated by the fact that with abundant food the animals reproduced very rapidly (see Chapter 5). Air Water Lift screening was expensive, labour intensive (socks had to be emptied daily) and relatively inefficient.

3.5.2.1.2 OVERFLOW SCREEN

A large self-cleaning wedge bar screen of mesh size 500 mm was installed in a 1,000 m^3 plankton pond and a 18 mm Warman Pump was used to deliver a volume of about 110 m^3 per hour continuously over the screen in a recycling system (see Chapters 2 and 6). This method was effective in screening adult *A.franciscana*, involved little labour and assisted in the aeration of the pond. *A.franciscana* collected on the screen were directed by a pipe at the bottom corner of the screen into the adjacent *A.franciscana* pond.

3.5.3 Elimination Of *F.salina*

The predatory protozoan *F.salina* periodically appears in numbers as high as 20/mL in saltfield brines (internal Dampier Salt monitoring programme). When brine containing *F.salina* was fertilised with soluble fertiliser and aerated, a bloom of planktonic microalgae quickly developed followed by a bloom of *F.salina* and the complete collapse of the microalgae population (see Section 3.4).

A variety of methods aimed at eliminating, or controlling, infestations of *F.salina* were trialed and are discussed below.

3.5.3.1 Methods

3.5.3.1.1 EXPERIMENT 3.5.1: THE EFFECTIVENESS OF DIATOMACEOUS EARTH FILTRATION IN REMOVING F.SALINA FROM FIELD BRINES INTENDED FOR MICROALGAE MASS CULTURE.

The possibility of filtering *F.salina* from the brine was initially considered as a simple method of eliminating this organism. Preliminary laboratory trials had revealed that *F.salina* could be completely removed from small volumes by filtration through absorbent cotton wool but not by non-absorbent cotton wool. Diatomaceous-earth filtration is effective for large volumes of water and it was hoped that it would effectively remove *F.salina* from brine.

Brine of density about 1.10 g/mL was used to fill four by 1,000 L tanks. The brine in two of the tanks was unfiltered and in the other two had passed through a diatomaceous-earth filtration system prior to entering the tanks. All brine was then fertilised with 0.05 g "Thrive" fertiliser/L and each tank inoculated with 2 L of *D.salina* culture, from an earlier experiment (Section 3.4, Experiment 3.4.2), that had been filtered through absorbent cotton wool to remove *F.salina* and clumps of cyanobacteria. This brine contained 39,000 *D.salina* cells/mL and no *F.salina* were present on GF/C filter papers through which 1 L of brine had been filtered. The experiment ran for two weeks with daily monitoring of both the *D.salina* and *F.salina* populations.

3.5.3.1.2 EXPERIMENT 3.5.2: DETERMINATION OF LETHAL CONCENTRATION OF CHLORINE FOR *F.SALINA*.

It appeared, from the results of Experiment 3.5.1, that removal of all *F.salina* by filtration from large volumes of brine was difficult. An alternative method of chlorinating all incoming brine was then considered. The minimum dosage of chlorine needed to eliminate *F.salina* from the brine column was determined by the following method.

50 mL of brine containing a concentration of about 48 F.salina/mL was added to each of eight vials. A standardised solution of Sodium Hypochlorite was prepared and added to the brine to give chlorine concentrations ranging upwards in 10 ppm increments from 0 to 40 ppm chlorine, duplicated. The vials were well mixed by shaking and a 0.5 mL subsample was taken immediately. All animals in the subsample were counted and recorded as alive or dead (on the basis of movement and buoyancy).

3.5.3.1.3 EXPERIMENT 3.5.3: DETERMINATION OF LETHAL TEMPERATURE FOR *F.SALINA* IN LABORATORY BIOASSAY.

A third option of eliminating *F.salina* was to pasteurise all incoming brine. The minimum lethal temperature required to eliminate *F.salina* was determined by the method outlined below.

Saltfield brine of density about 1.07 g/mL and containing about 20 *F.salina*/mL was placed in stoppered test tubes which were placed in a beaker of water on a hot plate. A thermometer suspended in the water was used to determine water temperature. Test tubes were removed from the beaker at 5°C intervals and placed on a rack for cooling. The brines were then examined for *F.salina* which are visible to the naked eye and they were recorded as living or dead. Dead *F.salina* sink to the bottom of the test tube and are completely still. Living *F.salina* remain suspended in the brine column and are motile.

3.5.3.1.4 EXPERIMENT 3.5.4: THE EFFECT OF BRINE DENSITY ON THE SURVIVAL OF *F.SALINA*.

Microalgae, particularly *D.salina*, can be cultured in a range of brine densities. A knowledge of the brine density preferences for *F.salina* may have a bearing on the culture conditions chosen for the algae.

50 mL of brine from each of the nine brine transfer points in the salt field was added to separate vials in an unreplicated range-finding experiment. 5 mL of *D.salina* culture containing 2,550 cells per mL was added to each flask as food in addition to 5 mL of brine containing about 1,600 *F.salina*/L (i.e. about 8 *F.salina*/flask). The population of *F.salina* was monitored weekly as described in Experiment 3.5.2.

3.5.3.1.5 EXPERIMENT 3.5.5: THE EFFECT OF *ARTEMIA* ON POPULATIONS OF *F.SALINA*.

An experiment was designed to determine the effect of *A.franciscana* on the population of *F.salina* with a view to using them in a biological screening process.

Five by 120 mL flasks were filled with brine containing 45 *F.salina*/mL. One pair of *A.franciscana* was added to each vial with the exception of a control flask. The population of *F.salina* was monitored after two days and six days. A small amount of *D.salina* culture was added daily to each flask to feed the animals. The experiment was then repeated using four flasks with one control containing no *A.franciscana*.

3.5.3.2 Results

3.5.3.2.1 EXPERIMENT 3.5.1: THE EFFECTIVENESS OF DIATOMACEOUS EARTH FILTRATION IN REMOVING *F.SALINA* FROM FIELD BRINES INTENDED FOR MICROALGAE MASS CULTURE.

There was no significant difference in either brine pH or density (g/mL) after two weeks (Table 3.5.1). The population growth of *F.salina* (Figure 3.5.2) shows that although the filtered brine was free of *F.salina* it was present in all tanks after ten days and rapidly expanded with a corresponding decrease in the population of *D.salina* (Figure 3.5.1). *D.salina* cells were clearly visible within the *F.salina*.

Table 3.5.1: Brine pH and density (g/mL) after two weeks in Experiment 3.5.1.

Treatment	pH		Density(g/mL)		n
	Mean	SE	Mean	SE	
1.Control	8.06	0.02	1.1002	0.00002	2
2.Filtered	8.08	0.02	1.0993	0.00006	2
Probability	0.45		0.25		

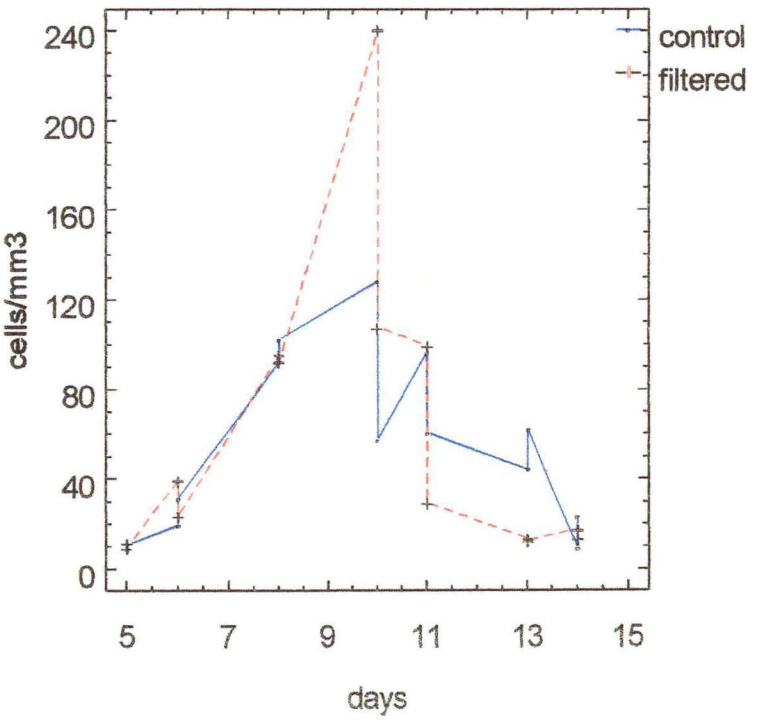


Figure 3.5.1. Population of *D.salina* (cellsX10^3/mL) in filtered and unfiltered brines over 14 days in Experiment 3.5.1.

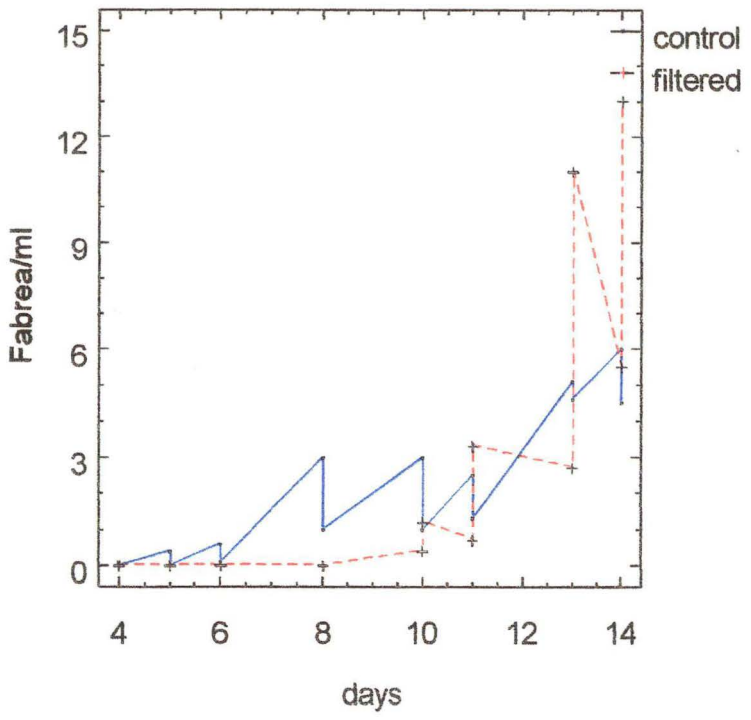


Figure 3.5.2. Population of *F.salina*/mL in filtered and unfiltered brine over 14 days in Experiment 3.5.1.

3.5.3.2.2 EXPERIMENT 3.5.2: DETERMINATION OF LETHAL CONCENTRATION OF CHLORINE FOR *F.SALINA*.

In Experiment 3.5.2 all animals in vials containing 10 ppm chlorine or higher were dead and control animals were alive.

3.5.3.2.3 EXPERIMENT 3.5.3: DETERMINATION OF LETHAL TEMPERATURE FOR *F.SALINA* IN LABORATORY BIOASSAY.

In Experiment 3.5.3 all *F.salina* in test tubes subjected to temperatures of 50°C or higher were dead and all those in brines of 45°C or lower were alive.

3.5.3.2.4 EXPERIMENT 3.5.4: THE EFFECT OF BRINE DENSITY ON THE SURVIVAL OF *F.SALINA*.

In Experiment 3.5.4 the population of *F.salina* throughout the brine density range 1.0425 g/mL to 1.2025 g/mL on days 0, 6 and 16 are shown in Figure 3.5.3. *F.salina* grew well at all brine densities up to 1.1143 g/mL with best growth at 1.085 g/mL. It was completely inhibited in brines of density 1.1553 g/mL after 16 days but survived for six days in brines at salting point (1.2023 g/mL).

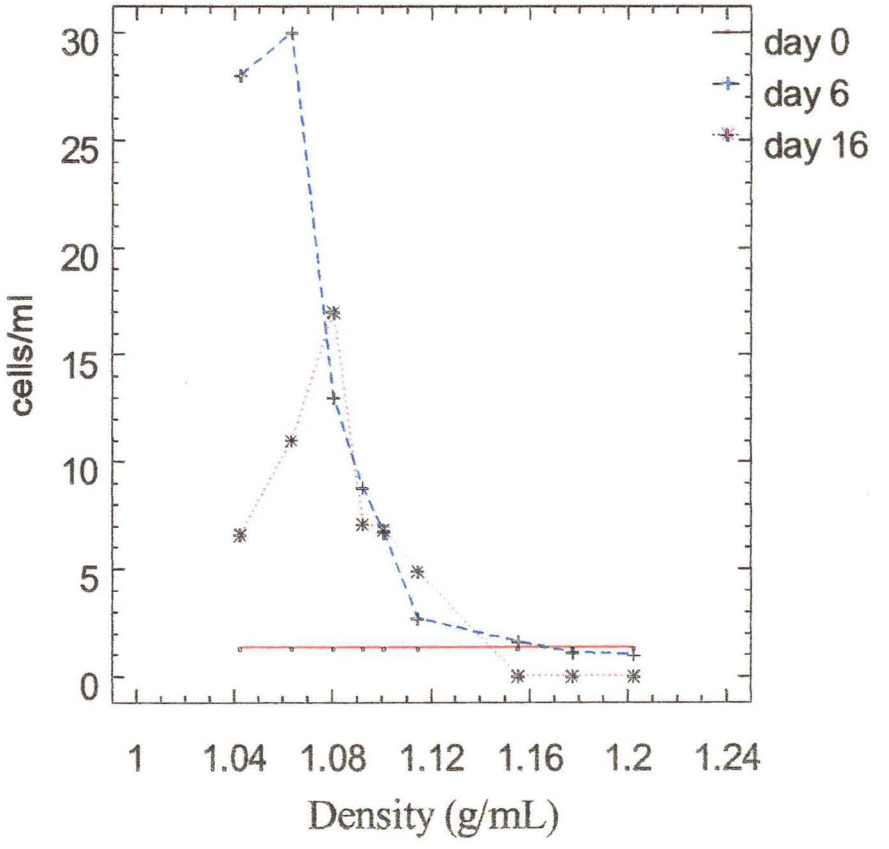


Figure 3.5.3. Effect of brine density (g/mL) on population of *F.salina* over 16 days in Experiment 3.5.4.

3.5.3.2.5 EXPERIMENT 3.5.5: THE EFFECT OF *ARTEMIA* ON POPULATIONS OF *SALINA*.

In Experiment 3.5.5 no *F.salina* were detected in any flask containing *A.franciscana* in both trials after six and four days respectively despite large and unchanged populations in the control beakers (Table 3.5.2).

Table 3.5.2: Population of *F.salina*/mL during trials

Treatment	Trial 1		Trial 2	
	Day 2	Day 6	Initial	Day 4
Control	9.6	10	38	26
replicate 1	8	0	38	0
replicate 2	2	0	38	0
replicate 3	4	0	38	0
replicate 4	10	0	38	0

3.5.3.3 Discussion

Protozoan contamination can devastate cultures in less than 24 hours, despite the use of sterilisation and ultrafiltration techniques and treatment of protozoan contamination remains difficult (De Pauw 1981 cited in De Pauw and Persoone 1988). Chapter 3.5 is devoted to developing practical solutions to problems caused by contamination and grazing of large-scale microalgae cultures by protozoans and *Artemia*.

In Experiment 3.5.1 there was rapid growth of *D.salina* in all tanks until about day eight (Figure 3.5.2). The populations then collapsed and there was a corresponding increase in the population of *F.salina* (Figures 3.5.1 and 3.5.2).

The use of diatomaceous-earth filtration was unsuccessful in completely eliminating *F.salina* from the brine column, although it did reduce the population to undetectable levels for about one week. It is apparent that any filtration system must remove 100% of the *F.salina* to be useful and this is difficult with large volumes of brine. It is possible that there was contamination from unfiltered brine housed in adjacent tanks although extreme care was taken to avoid this problem.

In Experiment 3.5.2, a concentration of 10 ppm chlorine was sufficient to kill all *F.salina*. It is likely that lower dosages than this would also be lethal but in large-scale test work where an 800 m^3 pond was dosed with chlorine using a V-notch chlorinator it was operationally difficult to continuously chlorinate incoming brine at chlorine levels much below 10 ppm. A slight excess would be used in any case to be certain of killing all animals. Outdoor trials conducted in 1,000 L tanks (unreported) revealed that chlorination was extremely effective in eliminating *F.salina*, and for that matter all the organisms in the brine column. The brine could then be inoculated with microalgae culture free of unwanted microorganisms but only after a period of at least four days had elapsed since chlorination. When inoculation was attempted

earlier than this there was no growth of *D.salina* even when chlorine levels were undetectable. It is possible that toxic byproducts from the chlorination process need time to dissipate.

Chlorination was found to be a very effective means of removing all microorganisms from the brine used to fill both experimental tanks and large ponds. A period of at least three days was found to be necessary before *D.salina* added to the brine would bloom. Presumably this time was necessary for the chemical effects of chlorinating brine to be reversed to the extent where the *D.salina* could tolerate conditions. Chlorine itself becomes undetectable within 30 minutes of brines being exposed to full sunlight. The elimination of all competitor microorganisms from the brine minimised problems of dominance and succession although in the longer term many species initially eliminated from the brine were found to be present in the ponds. With unlined clay based ponds it is possible that organisms such as cyanobacteria are protected from chlorine in the mud and later migrate into the brine.

In Experiment 3.5.4, *F.salina* initially grew in all the brines but with fastest growth at lower brine density. The optimum brine density for *F.salina* growth appears to be in brine of density about 1.0805 g/mL from the saltfield which is where the largest naturally occurring populations also occur (Figure 3.5.3). *F.salina* appears to be inhibited, in the longer term, in brine above about 1.155 g/mL density (consistent with natural occurrences) but survives quite well in brine of density 1.11 g/mL which would be close to the maximum brine density considered for the culture of *D.salina* used as food for *A.franciscana* (see Chapter 5.1 and 6). The brine originated from different ponds so it is possible that a factor other than salinity was responsible for the inhibition of *F.salina.*

A.franciscana were effective in eliminating *F.salina* from the brine column in Experiment 3.5.5 (Table 3.5.2) although the mechanisms for this elimination are unclear. It may be possible that the *F.salina,* which are soft bodied can be ingested by *A.franciscana* even though they are 170 mm long which is above the maximum 50 mm particle size capable of being ingested by *A.franciscana* (Sorgeloos et al., 1986). The other possibility is that *A.franciscana* render conditions unsuitable for *F.salina,* perhaps by out-competing them for the limited food supply.

The effectiveness of *A.franciscana* in eliminating *F.salina* suggests that needed make-up brine should be added to the pond containing *A.franciscana* and not to the pond containing the microalgae food supply. *A.franciscana* would be screened from this brine as it passed from the *A.franciscana* pond to the microalgae pond. The other advantage in adding makeup brine to the *A.franciscana* Pond is to maximise the difference in hydrostatic head driving the flow of brine through the screening system.

The experiments described in Chapter 3.2 demonstrated the importance of optimising physical conditions in order to maintain cultures of microalgae. If conditions were not optimised undesired competitors, particularly benthic diatoms and cyanobacteria, tended to dominate. As mentioned previously chlorination was found to be the most practical and effective method of initially eliminating all competitor microalgae from the brine. This is consistent with Richmond (1986) who states that the two basic methods of maintaining unialgal cultures are to optimise conditions for the desired microalgae or provide conditions which selectively promote or inhibit specific algae.

CHAPTER 4: THE GROWTH RESPONSE OF *A.franciscana* TO FOOD LEVEL

4.1 Growth, Survival and Food Conversion for A.franciscana Fed Mass Cultured Dunaliella spp. *in Field Brine.*

4.1.1 Introduction

Methods for extensive culture of Artemia spp. in salt fields in several countries have been well reported (Sorgeloos et al., 1986; Jumalon et al., 1987; Rodriguez et al., 1987; Tackaert and Sorgeloos, 1991). Large scale semi-intensive culture is not practised widely although techniques for intensive culture on a small scale are well understood Sorgeloos et al., 1986; Tackaert and Sorgeloos, 1991).

Techniques for large scale pond culture of D.viridis and D.salina in saltfield brines were developed in Chapter 3. These cultures were intended as food for Dampier strain A. franciscana grown intensively in large outdoor ponds, however, appropriate feeding strategies had to be determined.

The growth responses of various strains of A.franciscana to a range of microalgae have been well researched although the nutritional value of cultured microalgae can vary greatly with species and culture conditions (Brown et al., 1989; Jeffrey and Garland, 1988). Some authors (Mason, 1963; Johnson, 1980; Rowsowski, 1989) used A.franciscana from San Francisco Bay while others (Reeve, 1963a,b,c; Brune and Anderson, 1984, 1989) used A.franciscana from the Great Salt Lakes. None of these authors used D.salina or D.viridis as a food source although D.tertiolecta, a lower salinity species was used in some studies (Mason, 1963; Johnson, 1980). Most of the published studies used axenic laboratory cultures, yet the value of bacteria in the diet of Artemia has been well documented (Gibor, 1956; D'Agostino and Provasoli, 1968; Douillet, 1987).

An examination of the published data reveals great discrepancies. The two major studies on growth efficiencies (Mason, 1963; Reeve, 1963a) yielded results ranging from 4 to 5% (Mason, 1963) up to peak efficiencies of 79% (Reeve, 1963a). The effect of competition on growth and efficiencies are not well understood even though a mathematical simulation has been produced by Brune and Anderson (1989), using the data of Reeve (1963a) to explain size diversity in Artemia cohorts. Most published growth studies on Artemia used communal animals (Gibor, 1956; Reeve,

1963a; Johnson, 1980; Rowsowski, 1989) while Mason (1963) used solitary animals for his growth efficiency study. It was difficult, therefore, to use published information with confidence to determine optimum feeding rates for Dampier strain A.franciscana fed Dunaliella spp. mass cultured in outdoor ponds.

In this study the growth responses of A.franciscana fed Dunaliella spp. were determined for a range of feed rates (cells/animal/day) for solitary or communal cultures and for a range of A.franciscana densities with a fixed food input (cells/animal/day). The solitary cultures were undertaken to determine responses in the absence of intraspecific competition which is a factor in communal culture systems.

4.1.2 Methods

4.1.2.1 Dunaliella *Culture*

The Dunaliella spp. were cultured using techniques developed and described in Chapter 3. The concentration of cells in mass algae cultures was determined from haemocytometer counts. An average of five counts of cells contained in a volume of 0.1 mL was used to calculate the volumes of culture to be added to each feeding treatment. This volume was then dispensed into the A.franciscana culture brines with an adjustable 5 mL auto-pipettor adjustable in 0.01 mL increments.

4.1.2.2 A.franciscana

The A.franciscana used in the experiment were obtained from the Dampier Salt Limited saltfield at Dampier in the NW of Western Australia. This saltfield was first inoculated with A.franciscana in 1973 using cysts obtained from the Shark Bay saltfield which had in turn apparently been inoculated with A.franciscana several years previously (Burnard and Tyler, 1994).

Experimental animals were obtained by hatching cysts collected from the shores of the brine concentrating ponds in seawater (density 1.025 g/mL, Appendix 3). Hatched nauplii were captured using Pasteur pipettes and placed on a cavity slide before being temporarily stranded by withdrawing brine from the cavity. They were then measured from the nauplius eye to the telson under a dissecting microscope fitted with a calibrated eyepiece graticule, and transferred to beakers containing brine of density 1.10 g/mL, identical to the brine containing the cultured microalgae. Larger experimental animals were measured in the same way with the brine slowly

withdrawn as the animals swam forwards to ensure they were stranded in an extended position.

4.1.2.3 Dry Weight Determinations

The length to dry weight relationship for *A.franciscana* was based on the equation of Nimura, 1980. Dry Weight (ng) = 857xL(mm)^3. This equation is accurate for data published by Reeve, 1963a and for seven *Artemia* with lengths ranging from 0.444 mm (nauplius) to 10.45 mm tested from the Dampier Salt Limited strain of *A.franciscana* used in the experiments (Appendix 4).

The method for determining the dry weight of *D.viridis* is explained in Appendix 4. This weight of 6.7X10^{-11} g/cell was also used for the similar sized *D.salina*. The Specific Gravity of *Dunaliella spp.* is determined largely by the intracellular glycerol content which increases with increasing salinity to protect the cells from osmotic stress in hypersaline brine (Borowitzka 1974). Personal observations from centrifuge concentration of microalgae cells in brine indicate that the density (g/mL) of *Dunaliella spp.* living in brine of density 1.10 g /mL is very close to that of the brine, i.e. they will not concentrate readily in the bottom of centrifuge tubes after spinning as they will in brines of lower density (g/mL).

4.1.2.4 Experiment 4.1: The Growth Response of Communal A.franciscana *to Food Ration of* D.viridis.

Ten newly hatched nauplii were added to 50 mL plastiglass beakers filled with brine of 1.10 g/mL. There were four replicate beakers for each treatment encompassing five rations of 1X10^5 to 2X10^6 *D.viridis* cells/animal /day.

Animals were batch fed at five feeding levels in quadruplicate. All animals were counted and transferred daily to a second series of beakers containing fresh brine and the appropriate amount of algae per animal dispensed on a per animal basis. The experiment lasted for fifteen days.

4.1.2.5 Experiment 4.2: The Growth Response and Efficiency of Solitary A.franciscana *in Relation to Food Ration of* D.salina.

Conditions were similar to Experiment 4.1 except that each replicate beaker contained only one nauplius and feeding rates ranged from 5*10^5 to 8*10^6 *D.salina* cells/animal/day for 14 days. Growth efficiencies were calculated on the basis of

biomass gain (dry weight)/food consumed (dry weight) (Appendix 4).

4.1.2.6 Experiment 4.3: A Comparison of the Growth Response of A.franciscana *in Solitary and Communal Culture to* D.salina.

In contrast to Experiments 4.1 and 4.2 this was a synchronous comparison of cultures stocked with 1 nauplius per 50 mL culture (40 beakers per feed rate) and ten nauplii per 500 mL culture (four beakers per feed rate). For each type of culture there were two feed rations ($1X10^6$ and $2X10^6$ *D.salina* cells/animal/day) in a factorial design.

The beakers were organised in four spatial blocks, each comprising one replicate of each communal treatment and ten replicates of each solitary treatment. Beakers were randomised within blocks on the bench. The appropriate numbers of newly hatched nauplii were added to each beaker and a subsample of 20 nauplii from the same batch of nauplii was measured to estimate average initial length.

The animals were batch fed daily with laboratory *D.salina* culture in the same brine as the outdoor cultures described above but housed in 500 mL beakers agitated in an environmental chamber with temperature 25°C, light intensity 1500 lux and a 12 h:12 h light/dark cycle. Algal densities in the culture to be added to the *Artemia* culture were determined from ten individual haemocytometer counts and the appropriate volumes dispensed to each beaker. Culture brine was removed with a screened syringe prior to the addition of food to compensate for the volume of algal culture added. Food input was constant per animal. All *Artemia* were measured at the end of the trial after ten days.

4.1.2.7 Experiment 4.4: The Population Response of Various Populations of Communal A.franciscana *to Constant Food Rations of* D.salina.

Experiment 4.1 indicated that an adult *A.franciscana* required a minimum of 500,000 *D.viridis* cells/day to survive. The population of highly competitive communally cultured animals is likely to fluctuate with food ration with individual animals receiving close to the minimum amount of food required for survival. This experiment was established to monitor the population change over five weeks of different initial populations of highly competitive communal *A.franciscana* receiving the same amount of *D.salina*. Reproductive output was also monitored.

Ten million cells of laboratory cultured *D.salina* were dispensed daily to each of twelve plastic beakers, containing 500 mL of brine of density 1.08 g/mL. Populations of 60, 40 and 20 *A.franciscana*/beaker were established in quadruplicate.

The animals were all initially about 6 mm long and were added to the beakers from a large stock culture with all animals hatched from a single batch of cysts a week earlier.

The cultures were monitored weekly by sieving culture brine through a 500 mm mesh which retained adults but allowed nauplii, cysts and faecal material to pass through. The adults were then returned to clean beakers with fresh brine and the waste brine was filtered through a GF/C glass fibre filter paper which caught all cysts and nauplii. These were then counted under a dissection microscope and not returned to the beakers. Although much care was taken to minimise damage to screened adults the transfer did cause mortalities. The cultures were therefore left unchanged for the final two weeks of the five week experiment. All animals were measured under a dissection microscope equipped with an eyepiece graticule at the end of the experiment. Lengths were then converted to dry weights using the formula of Nimura (1980) (Dry Weight (ng) = 857 x length^3 (mm) (Appendix 4).

4.1.3 Results

4.1.3.1 Experiment 4.1: The Growth Response of Communal A.franciscana *to Food Ration of* D.viridis.

The growth response of communal *A.franciscana* in a single replicate from each treatment is shown in Figure 4.1.1. Growth rates and maximum lengths achieved were increased by higher feeding rates. The aberration on Day 15 when the lowest feeding level treatment had animals of greater mean length than the second lowest feeding level was caused by the deaths of two of the largest animals in Treatment 2 which lowered the overall mean. Day 15 data from all replicates in Table 4.1.1 clearly shows the distinct and highly significant separation of mean lengths. Highly significant differences in mean lengths were also apparent after only one day's feeding (Table 4.1.1).

Each beaker originally had ten communal animals and there were mortalities in all but three beakers during the course of the experiment and no reproduction. Mortalities in the two lowest feeding levels were higher than those in the three highest feeding levels (Table 4.1.1). At the end of the experiment surviving animals in the two lowest feeding levels appeared very inactive and it is probable that all of them would have died

if the experiment had been extended for even a few additional days.

Table 4.1.1: Mean lengths (µm) of communal *A.franciscana* after one day and 15 days feeding and transformed percent survival after 15 days in Experiment 4.1. A newly hatched nauplius is about 440 µm long.

			Mean Length (microns)± Std.Error		% Survival Mean±SE*
***D.viridis*/animal/day**		**No.**	**Day 1**	**Day 15**	
1.	100,000	4	760 +- 15^{a}	2880 +- 193^{a}	50±10.8 ^{a}
2.	200,000	4	770 +- 11^{a}	3463 +- 435^{a}	40±7.1^{a}
3.	500,00	4	880 +- 22^{b}	3936 +- 116^{b}	85±6.5^{b}
4.	1,000,000	4	861 +- 16^{b}	5282 +- 34^{c}	87.5±4.8^{b}
5.	2,000,000	4	920 +- 19^{b}	6666 +- 104^{bc}	90±5.7^{b}

Means sharing a common superscript are not significantly different.
*Data were transformed by Arcsin sq.rt prior to analysis and subscripts are based on these data.

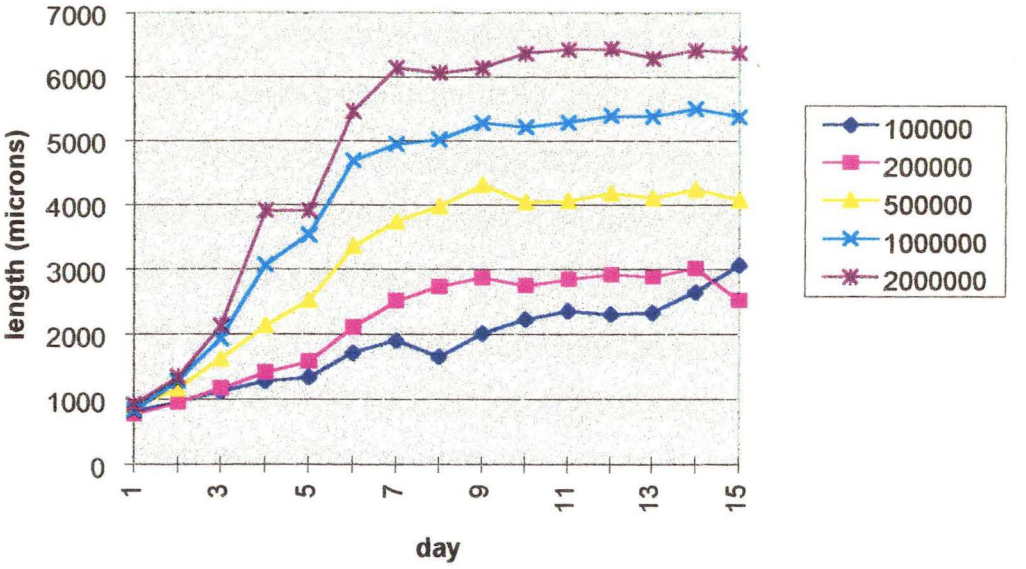


Figure 4.1.1. Growth response of a single replicate of communal *A.franciscana* to various inputs of *D.viridis* as cells/animal/day in Experiment 4.1.

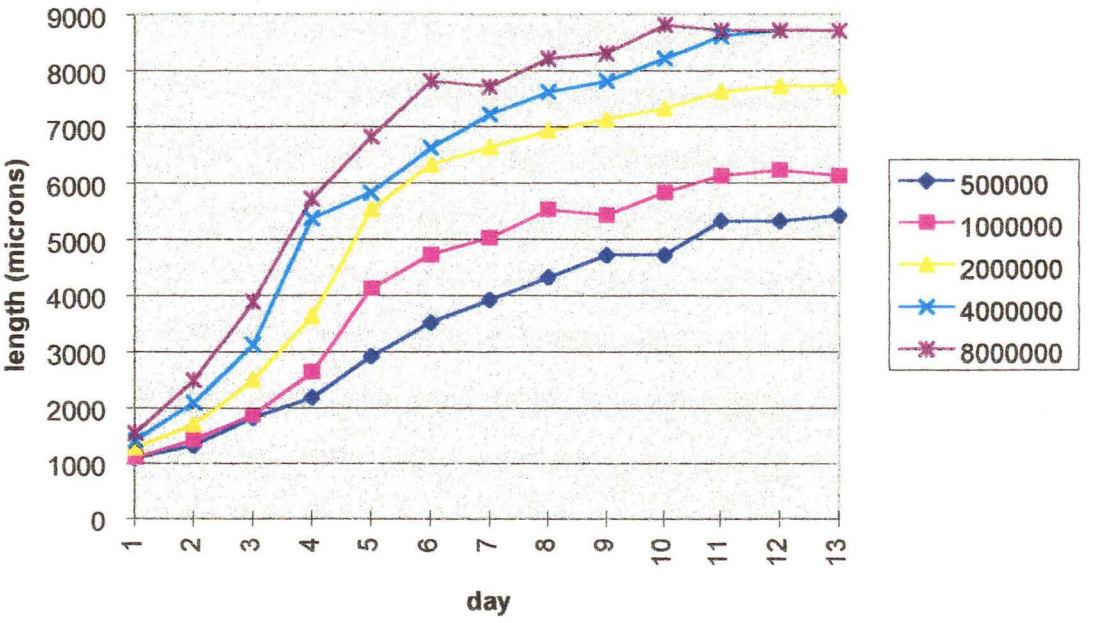


Figure 4.1.2. Growth response of solitary *A.franciscana* to various inputs of *Dunaliella salina* as cells/animal/day in Experiment 4.2.

4.1.3.2 Experiment 4.2: The Growth Response and Efficiency of Solitary A.franciscana *to Food Ration of* D.salina.

The growth response of solitary *A.franciscana* to level of *D.viridis* food is shown in Figure 4.1.2 and the overall size differences on Day 14 at the end of the experiment are shown in Table 4.1.2. These data exclude animals that died during the course of the experiment (i.e. one from Treatments 1, 2, and 4 and two animals from Treatment 5). The means plot (95% LCD) for overall growth efficiencies for the two week trial are included in Figure 4.1.3 and Growth Efficiencies through time in Table 4.1.3. There was a clear pattern of decreasing efficiency with increased food input and maximum growth efficiencies in the six to ten day period. These efficiencies encompass the period of growth from nauplius through until the plateau size achieved for each food level (Figure 4.1.2).

Table 4.1.2: Mean lengths (mm) and 95%LSD intervals for *A.franciscana* fed *D.salina* for 14 days in Experiment 4.2.

Treatment	Mean	SE	n
500,000 cells/animal/day	5206[a]	117	3
1 million cells/animal/day	5949[b]	364	3
2 million cells/animal/day	7590[c]	100	4
4 million cells/animal/day	8657[d]	115	4
8 million cells/animal/day	8488[d]	340	2
ANOVA probability	0.0000		

Means with common superscripts are not significantly different (p>0.05).

Table 4.1.3: Growth efficiencies (dry weight gain/consumed food dry weight*) of a single replicate in Experiment 4.2.

Treatment	Day 1-5	Day 6-10	Day 11-14
500,000 cells/animal/day	7%	44%	30%
1 million cells/animal/day	5.6%	32.3%	21.6%
2 million cells/animal/day	10.7%	31.5%	18%
4 million cells/animal/day	5.6%	22.7%	17.5%
8 million cells/animal/day	5.4%	11.3%	4.1%

*consumed weight calculated on the basis of remaining food in beakers prior to feeding with assumption of zero microalgal productivity.

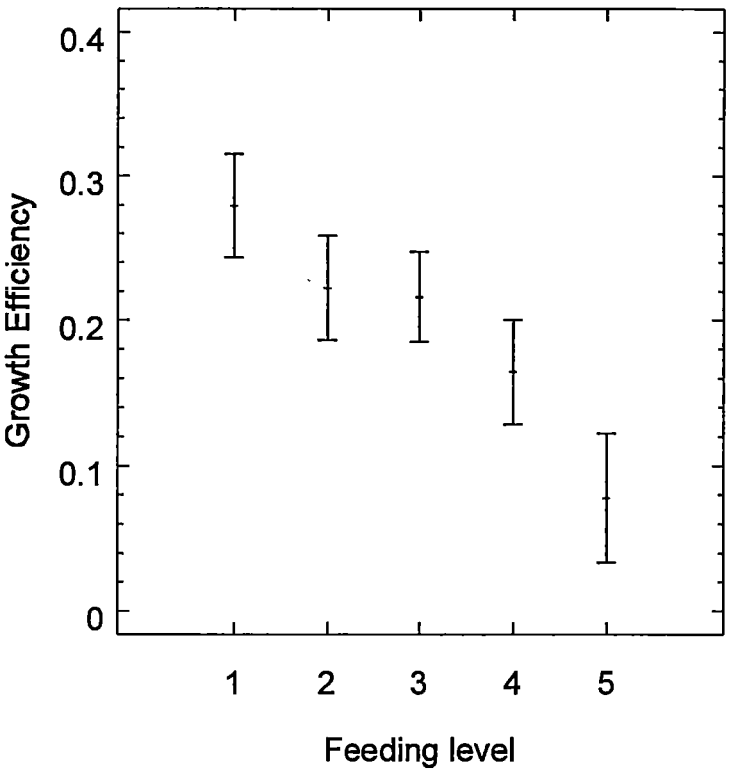


Figure 4.1.3. Means plot (95% LSD) for overall growth efficiencies (dry wt.animal/dry wt.food consumed) in Experiment 4.2 at five feed rations (1=500,000; 2=1,000,000; 3=2,000,000; 4=4,000,000 and; 5=8,000,000 cells/animal/day).

4.1.3.3 Experiment 4.3: A Comparison of the Growth Response of A.franciscana *in Solitary and Communal Culture, to Food Ration of* D.salina.

The 2 Way ANOVA comparing the final sizes of animals from communal and solitary culture for different feed inputs is presented in Table 4.1.4. Growth rates and achieved final lengths were not significantly affected by solitary or communal conditions. Food level did, however, cause highly significant size differences. The absolute ten day size ranges are presented in Table 4.1.5 and reinforce the ANOVA results. The stress exhibited by solitary animals in Experiment 4.2 once sexual maturity was reached was avoided by terminating the experiment after ten days when some animals were just becoming sexually mature.

Table 4.1.4: ANOVA table of animal lengths (μm) after ten days feeding in Experiment 4.3.

TREATMENT		Mean	SE	n	P
Culture	1.Solitary	8072[a]	131	8	0.94
A	2.Communal	8059[a]	131	8	
Food	1 million cells/animal/day	7647[a]	131	8	<0.001
B	2 million cells/animal/day	8483[b]	131	8	
Interaction AB		0.48			

Means with common superscripts are not significantly different. SE based on mean square error.

Table 4.1.5: Comparison of *A.franciscana* size ranges after ten days feeding in Experiment 4.3.

Treatment	Length (mm)
Solitary (1 million cells/animal/day)	6009-9358
Solitary (2 million cells/animal/day)	6895-10047
Communal (1 million cells/animal/day)	6107-9161
Communal (2 million cells/animal/day)	6895-10343

4.1.3.4 Experiment 4.4: The Population Response of Various Populations of Communal A.franciscana *to Constant Food Ration of* D.salina.

The Mean ±95% LSD intervals for final (five week) *A.franciscana* length (mm); total dry weight (mg), and population/beaker after five weeks are shown in Table 4.1.6. The animals in the treatment with initially 60 animals were significantly longer and the population was still significantly higher than in the other two treatments despite a steady population decline to a mean of 69 animals/L corresponding to a total mean dry weight loss of 9.2 mg/L. The other treatments experienced weight gains of about 8.2 mg/L. There was some recruitment in Treatment 3 which initially had 40 *A.franciscana*/L and finally had a mean of 41.5/L large enough to be retained by the 500 mm screen.

The Mean ± 95% LSD Intervals for total reproductive output over the five week experiment is presented in Table 4.1.7. Cyst production was not significantly different between treatments (p=0.094) but overall reproductive output was significantly (p=0.0037) lower in the treatment which initially had 120 animals/L. The population in these beakers was declining as the animals starved.

Table 4.1.6: Mean ± SE for *A.franciscana* population; animal lengths (mm); final dry weight (mg) and; dry weight change (mg) after five weeks in Experiment 4.4 with fixed *D.salina* input (1 million cells/L/d).

Initial Pop./L	No.	Population/ beaker after five weeks	Mean Length (mm)	Final Dry Weight /beaker (mg)**
40	4	41.5±9[a]	8.1±1.44[a]	5.1±1.34[a]
80	4	41.5±3.86[a]	8.46±0.96[a]	5.6±1.06[a]
120	4	69±5[b]	12.4±0.88	16.6±2.96[b]
ANOVA p		0.02	0.09	0.045

*Means with common superscripts are not significantly different.
** Initial dry weights (6 mm animals) were 7.4 mg, 14.8 mg and 22.2 mg for populations of 40, 80 and 120/L respectively. The final dry weight data did not include weight of reproductive output..

Table 4.1.7: Mean ± SE for *A.franciscana* total reproductive output and cyst production/L over five weeks in Experiment 4.4.(ANOVA was conducted on transformed (√)data but Means for untransformed data are presented.

Initial Population/ L	No.	Cysts	Total Reproduction
40	4	169.6±67.8	364.6±80.6[a]
80	4	117.6±23.66	314.6±58.2[a]
120	4	45±17.56	69±17.4[b]
ANOVA p		0.094	0.0037

Means with common superscripts are not significantly different.

4.1.4 Discussion

A clear pattern of both increased growth rates and maximum length achieved was evident as food ration increased for communally cultured animals in Experiment 4.1 (Figure 4.1.1, Table 4.1.1). It is possible, however, that even higher growth rates and sizes would have been achieved with higher food input and this was confirmed in Experiment 4.2. The maximum achieved size of over 8.5 mm after 15 days can be compared to the largest size achieved by the best strain (Margherita di Savoia, Italy) of the 17 strains investigated by Tobias et al. (1980) in the St. Croix artificial upwelling-mariculture system after 15 days. The Shark Bay strain (original Dampier strain) in the upwelling study was slightly smaller at a mean of 6.82 mm.

Large differences in animal sizes between treatments were apparent as early as Day one but these differences can be ascribed mainly to food concentration as excess *D.viridis* cells were present in all beakers at this time. A distinct size difference was apparent between animals in the lowest two treatments and animals in the upper three food densities. This indicates that a minimum food concentration of 10,000 cells/mL (Treatment 3) is required for good growth of the early stages of *A.franciscana* which are relatively inefficient filter feeders (Schrehardt, 1987). This is less than half of the optimal 25,000-30,000 cells/mL overall feeding rate for all instars recommended by Reeve (1963) for *Phaedactylum tricornatum*. However, that diatom has an unusually high mannose content and is generally not considered a highly nutritious microalgal feed for zooplankton (Brown et al., 1989; G.Maguire, personal communication 09/95). It is also a much larger organism with a dry weight of 0.0108 mg/million cells (Reeve, 1963a) giving each cell a dry weight about ten times higher than that calculated for *D.salina* (Appendix 4). The two lowest feeding levels also experienced the greatest mortalities (Table 4.1.1) and surviving animals within these treatments appeared extremely weak at the end of the trial. These two feeding levels can be considered too low to support adult *A.franciscana*.

Experiment 4.2 was conducted with solitary animals to avoid competition and obtain accurate growth efficiencies. The upper range of feeding levels was extended to determine the maximum growth response and this was achieved with 4 million cells per animal per day (Figures 4.1.2 and Table 4.1.2). The growth rates were similar to those found by Mason (1963) who used solitary animals fed on *D.tertialecta*. The death of animals and the sex of survivors may have influenced the results. Both surviving animals in the $8X10^6$ cells/animal/day feeding rate were males and reached maximum size on Day 11. They appeared to be in great behavioural stress and were clasping their lower bodies with their claspers for several days before dying on Day 13. All three

surviving animals at the lower feeding rate of $4X10^6$ cells/animal/day were female. Females attain a greater length than males and did not appear to suffer the same degree of stress from being in solitary culture. Although the experiment could be confounded to some extent by sex differences they only played a part in the latter stages of the experiment when the animals were mature.

The lack of competition enabled growth efficiencies to be determined. Growth efficiencies of one replicate through time are presented in Table 4.1.3 and show a pattern of maximum efficiencies, corresponding to maximum growth rates in the six to ten day period. The other exhibited pattern at all times was decreasing efficiency with increased food ration. A means plot of overall growth efficiencies is included in Figure 4.1.3 and shows decreasing efficiencies with increased food level. This is consistent with reports in the literature and is possibly related to the time the food actually spends in the gut. Both Reeve (1963) and Bossuyt et al. (1980) state the importance of continuous rather than batch feeding for this reason. In cases of extreme overfeeding the food can actually pass through the gut so rapidly that little is digested and growth rates are retarded (Nimura, 1980). Growth efficiencies ranged from 28% for animals fed 500,000 *D.salina* cells per day down to 8% for animals receiving eight million cells. The highest growth efficiencies achieved with the lowest feeding rate must, however, be considered in conjunction with growth rates which were lower than those of animals at higher feeding rates. The experiment extended to a total of 28 days and after this time animals in the 500,000 cells/animal/day treatment were still immature while animals at higher feeding levels had become sexually mature. Feeding levels of between 1 and 2 million cells (0.065 - 0.13 mg dry weight) per animal per day can therefore be considered optimal in terms of balancing growth efficiency against growth rates and increasing the certainty of *Artemia* reaching sexual maturity. At this feeding level, growth efficiencies are about 22% and maximum cell concentrations are between 2 and $4X10^4$ cells/mL in a 50 mL beaker. This encompasses the 2.5 to $3.0X10^4$ cells/mL optimum food concentration recommended by Reeve (1963). A feeding level of 2 million cells per animal per day equates to a dry weight of 0.14 mg which is slightly less than the 0.2 mg dry weight of wheat bran and *Ulva* used by Viera (1987) after Day 3. It is less than the 1 mg dry weight per animal per day recommended by Douillet (1987) for cereals.

Experiment 4.3 was undertaken primarily to assess the effect of competition on *A.franciscana* growth rates in order to assess the relevance of the findings of Experiment 4.2 to animals in communal culture. The high degree of size variation exhibited by communal animals in Experiment 4.1 (Table 4.1.1) and the

smaller variation between solitary animals in Experiment 4.2 (Table 4.1.2) tended to confirm the findings of Brune and Anderson, 1989, who found that size variation is great when animals are competing for limited food and less without competition. This needed to be confirmed with a concurrent experiment encompassing both solitary and communal animals with a high degree of replication.

There was no significant difference in animal length between solitary and communal culture although food quantity caused highly significant differences (Table 4.1.4). The experiment was unavoidably confounded by differences in volume between treatments but food and animal concentrations were identical and there was no reproduction until Day 10 when the experiment was terminated. The growth efficiencies determined in Experiment 4.2 are therefore likely to be relevant to communally cultured animals. There was a high and similar degree of variation in animal lengths in both solitary and communal culture at both feeding levels (Table 4.1.4, Table 4.1.5). The similarity between means and variation in animal length at each of the two feeding levels with either communal or solitary culture was as high or higher within each type of culture conditions as it was between culture conditions. This seems to contradict the model of Brune and Anderson, 1989, that large size variation between individual animals within a treatment are due to competition rather than genetics. The experimental animals originating from the saltfield appear to be intrinsically variable.

The above experiments indicated an optimal feeding rate of between $1X10^6$ and $2X10^6$ *Dunaliella spp.* cells/animal/day giving mean growth efficiencies of about 25% (dry weight/dry weight). In a large communal culture, however, unless population expansion was prevented, it would be difficult to sustain. With constant input of food the population of animals would be likely to fluctuate with food availability and individual animals under severe competition would be receiving low levels of food. This aspect was investigated in Experiment 4.4.

Despite a decline in both number of animals and total weight/L the initially 120 animals/L treatment with a final mean of 69 animals/L had significantly higher final adult populations than the other treatments and the animals were larger. This was because, unlike the other treatments, they were still almost all first generation and did not contain as many immature animals which that had grown to a size large enough to be retained by the screen during the final two weeks of the experiment when the brines were not screened (Table 4.1.6).

Total reproductive output during Experiment 4.4 (Table 4.17) was significantly higher ($p=0.004$) in treatments beginning with 40 and 80 *A.franciscana*/L that had similar populations of about 40/L after

five weeks than in the treatment that began with 120 animals/Landwhich still had a mean of 69 animals/L after five weeks. This was presumably caused by a lack of food in the 120 animal/L treatment which had a population that was declining due to starvation. The effect of food level on reproductive output is the subject of Chapter 5.1. The population of the initially 120 animals/beaker treatment probably would have declined further had the experiment been extended. The population of animals in the other treatments consisted of a number of generations of adults and developing animals of all sizes between nauplius and adult by the end of the experiment. Despite this complication it appears, when the continuing decline of the highest population treatment is considered, that the ten million *D.salina* cells fed each day is sufficient to support about 20 adult animals which would each receive about 500,000 cells per day. Although cyst production was not significantly different between treatments (p= 0.094, Table 4.17) there were much lower cyst numbers in the 120 animal/L treatment reflecting the general lack of reproductive output in this treatment.

4.1.5 Overview

The consistency between Experiments 4.1 and 4.2 and more importantly Experiment 4.3, suggest that in the absence of reproduction, food ration studies involving communal or solitary animals can be compared directly. However, the food source used is likely to be an important factor.

It appears from Experiment 4.4 that population density and reproductive output is ultimately determined by food supply

4.2 The Growth Response of A.franciscana *to Wheat Pollard and Mixed Pollard/*D.Salina *Diet.*

4.2.1 Introduction

Dunaliella spp., mass cultured in outdoor tanks is a good food for A.franciscana (Section 4.1) although some studies (Lavens and Sorgeloos, 1991) conclude that it is uneconomical to produce live microalgae food for large-scale Artemia culture. The alternative is inert food, and a number of types have been successfully trialed by other workers (D'Agostino and Provasoli, 1968; Viera, 1987; and Douillet, 1987). In their review of Artemia production in culture tanks, Lavens and Sorgeloos (1991) conclude that the two specific characteristics of Artemia correlated with a broad range of suitable diets are their non-selective filtration behaviour, facilitating the uptake of any sufficiently small particles; and the necessity of exogenous microflora as part of the diet. Preliminary feeding

experiments were undertaken at Dampier Salt using a variety of inert foods, including "Torula Yeast" and filamentous Rhizoclonium sp. algae. The latter was collected from the brine concentrating ponds of the Dampier Salt saltfield and dried and powdered prior to use. Both these foods were found to be suitable but relatively expensive or, in the case of Rhizoclonium, time consuming to collect in quantity. Other foods were then considered, particularly wheat pollard, which was available in bulk at a price of about A$0.25/kg.

A number of experiments were undertaken to assess the suitability of wheat pollard as a substitute or supplementary food for A.franciscana.

Experiment 4.5: The Growth Response of Solitary *A.franciscana* to Wheat Pollard, Mixed Wheat Pollard/*D.salina* and *D.salina* diet in 50 mL Laboratory Culture.

Experiment 4.6: The Growth Efficiency and Population Response of Communal *A.franciscana* In 500 mL Beakers to *D.salina* and Mixed *D.salina*/Wheat Pollard Diet.

Experiment 4.7: The Growth Efficiency and Population Response of Communal *A.franciscana* in 1,000 L Outdoor Tanks to *D.salina* and Mixed *D.salina*/Wheat Pollard Diet.

Experiment 4.8: The Growth Efficiency and Population Response of Communal *A.franciscana* in 1,000 L Outdoor Tanks to Wheat Pollard with and without *D.salina* Supplements.

4.2.2 Methods

4.2.2.1 Experiment 4.5: The Growth Response of Solitary A.franciscana *to Wheat Pollard, Mixed Wheat Pollard/*D.salina *and* D.salina *Diet in 50 mL Laboratory Culture.*

Viera (1987) recommended a feeding rate of 0.2 mg/animal/day for wheat bran but all experimental animals fed 0.2 mg wheat pollard/animal/day in a preliminary experiment died and appeared to have starved. A ration of 1 mg per animal per day, as recommended by Douillet (1987) for various inert foods, was then chosen as the feed ration for pollard as a sole diet.

The optimum food ration of two million cells per animal per day, as determined from earlier feeding trials (Section 4.1), was chosen for *D.salina* as a sole diet. This maximum rate is equivalent to 0.13 mg dry weight (Appendix 4).

Twenty 50 mL beakers were randomised on the bench and filled with 50 mL of filtered brine of density 1.08 g/mL. One premeasured first instar nauplius was added to each beaker. Five daily feeding treatments were applied in quadruplicate as follows:

Treatment	*D.salina* x10^6cells	Pollard (mg/day)
1.	2.0 (0.13 mg dry weight)	0
2.	1.5 (0.098 mg)	0.25
3.	1 (0.065 mg)	0.5
4.	0.5 (0.03 mg)	0.75
5.	0	1.0

The design was not based on a uniform dry matter input but recognised that *D.salina* was likely to be more nutritious than wheat pollard as indicated in preliminary trials. The *D.salina* culture was from a laboratory culture grown in 1.08 g/mL density fertilised saltfield brine. Algal cell numbers in five random haemocytometer fields each encompassing 25 µL volume were counted. The appropriate algal aliquot was dispensed into a clean beaker containing filtered field brine of 1.08 g/mL density. Wheat pollard (0.5 g) previously ground in a ball mill, was added to 500 mL of filtered brine of 1.08 g/mL density to give a concentration of 1 mg/mL. The pollard was evenly suspended using a magnetic stirrer. Pollard and *D.salina* were both dispensed with a 1 mL pippetor adjustable in 0.1 mL increments.

Animals were measured initially, after five days and after ten days (at the end of the trial). On days they were not measured they were transferred directly to new beakers except for Day 1 when they were difficult to see.

4.2.2.2 Experiment 4.6: The Growth Efficiency and Population Response of Communal A.franciscana *In 500 mL Beakers to* D.salina *and Mixed* D.salina*/Wheat Pollard Diet.*

Experiment 4.5 demonstrated that wheat pollard was an effective food for *A.franciscana*. Experiment 4.6 was designed to determine both growth efficiencies and the population size of communal *A.franciscana* culture to a diet of *D.salina* with and without wheat pollard supplements.

Approximately 500 nauplii were added to brine of density 1.08 g/mL in twelve 500 mL beakers. This population was expected to decline greatly during the course of the trial in all treatments to yield a final population supportable by the food level. The animals were daily decanted into clean beakers with fresh brine leaving most uneaten wheat and faecal material which could then be discarded. Three daily feeding treatments were applied at random in quadruplicate as follows:

***D.salina*/day**	**Wheat Pollard/day**
12.5 million cells	0
12.5 million cells	30 mg
12.5 million cells	60 mg

After eleven days, at the end of the trial, a total count of animals in each beaker and a measure of their size distribution were undertaken.

4.2.2.3 Experiment 4.7: The Growth Efficiency and Population Response of Communal A.franciscana *in 1,000 L Outdoor Tanks to* D.salina *and Mixed* D.salina*/Wheat Pollard Diet.*

Experiments 4.5 and 4.6 demonstrated that wheat pollard is an effective food for *A.franciscana* in laboratory culture. An experiment of similar design to Experiment 4.6 was undertaken in 1,000 L outdoor tanks to gauge the effectiveness of pollard as a food for large scale outdoor culture where waste products such as uneaten wheat and faecal material tend to remain in the culture vessel.

Microalgae, primarily *D.salina*, but including some *T.suecica* and other microalgae were mass cultured in a 1,500 m^3 plankton pond (density 1.08 g/mL) by methods developed and described in Chapter 3. A controlled flow of this culture was fed via an overhead manifold and a series of valves into twelve 1,000 L fibreglass tanks aerated with air delivered from an axial air blower through "Air/Water Wik" tubing to give a fine mist of bubbles. The tanks were equipped with overflow screens of mesh size 112 µm to retain all *A.franciscana* and their offspring. These screens were kept clear with air curtains.

The tanks were initially filled with seawater (density 1.024 g/mL) to facilitate the hatching of 10 g of cysts of hatchability about 25% that were added to each tank. This was designed to yield an initial population of 1,000 animals/L. Once the cysts had hatched microalgae culture brine of density 1.08 g/mL was passed through each tank. The treatments applied at random in quadruplicate were as follows:

1. Microalgae diet alone.
2. Microalgae plus 25 g wheat pollard/day.
3. Microalgae plus 50 g wheat pollard/day.

Flow rates were initially set at 1 L/minute through each tank. It soon became apparent, however, that this flow rate was too high and resulted in screens clogging and consequent overflow of tanks with loss of some animals. A system of rapid flow at 10 L/minute under constant observation was then adopted for a period of 30 minutes. This gave a daily turnover of culture brine of about 30% and was done before the addition of wheat pollard to minimise loss of food and clogging problems.

The loss of animals via overflow resulted in initial populations of nauplii being different. The initial populations were planned to be much greater than the populations that could finally be supported by the food level. This was still the case, even in tanks with the greatest loss of animals. The experiment was therefore continued with the expectation that final adult populations would ultimately be determined by food input level regardless of initial numbers and that mortalities would be much greater in tanks with higher initial populations.

Populations of *A.franciscana* were monitored weekly during the experiment by first mixing the brine with an oar and taking a 500 mL sample from each tank. Five subsamples were then taken and filtered prior to examination under a dissecting microscope. Unfortunately the data from the subsamples for each tank were highly variable and not useful. At the end of the experiment a total harvest of animals was made by filtering the culture brines through a 1 mm screen. Excess brine was squeezed from the animals which were weighed. The population of microalgae in culture brine fed into the tanks was monitored weekly by haemocytometer counts.

4.2.2.4 Experiment 4.8: The Growth Efficiency and Population Response of Communal A.franciscana *in 1,000 L Outdoor Tanks to Wheat Pollard with and without* D.salina *Supplements.*

This trial was undertaken in 1,000 L outdoor tanks to determine the effectiveness of wheat pollard as a food for *A.franciscana* with and without *D.salina* supplements. The algae was fed to the *A.franciscana* via a recycling system. It was hoped that the waste products of CO_2 and ammonia from *A.franciscana* would stimulate

the growth of the *D.salina* and the purified brine could be recycled.

The following model system was established from first principles and to my knowledge there are no reports of similar systems. Twelve by 1,000 L tanks were filled with brine of density 1.085 g/mL from the saltfield. Stockpile salt (virtually pure NaCl) was added to raise the density to 1.10 g/mL found to be ideal for *A.franciscana* culture in preliminary trials. The brine was aerated via perforated PVC pipe supplied with air from an axial air blower. All tanks were sterilised by the addition of 100 ppm chlorine added as liquid sodium hypochlorite. They were left for three days and then nine of the tanks were fertilised with "Aquasol" at a level of 0.02 g/L and inoculated with *D.salina* culture. These cultures became green within a week. Six of the inoculated tanks were arranged in pairs and connected by a screened overflow system. One tank of each of these pairs was used for *A.franciscana* culture and the other as a microalgae food and, water purification tank for the *A.franciscana*. 100 L of the algae culture was pumped daily into the *A.franciscana* tank and could backflow through a 112 µm screen kept clear with an air curtain. After one week, *A.franciscana* nauplii were added to the three clear tanks without algae; the three tanks paired to the adjacent algae tanks and; the three remaining unpaired tanks to give an initial population of about 100 animals/L in all nine tanks containing *A.franciscana*. The treatments in triplicate were therefore as follows with all *Artemia* tanks receiving 25 g of crushed wheat pollard per day:

1. No *D.salina* at any time.
2. Brine initially containing *D.salina* but with no further addition of microalgae culture.
3. Brine initially containing *D.salina* and 100 L of *D.salina* culture added per day.

The experiment ran for four weeks with *A.franciscana* populations, pH and brine density monitored on a weekly basis.

The total wet weight of animals was determined at the end of the trial by screening all culture brine through a 450 µm screen. The temperature range experienced by one tank was also monitored. The tanks were equally exposed to the sun, were of similar volume and received equal aeration.

4.2.3 Results

4.2.3.1 Experiment 4.5: The Growth Response of Solitary A.franciscana to Wheat Pollard.

The growth response of solitary A.franciscana to the various combinations of D.salina and wheat pollard (Figure 4.2.1) and the five day comparison of lengths (Figure 4.2.2) showed that initial growth rates of animals receiving only pollard were significantly retarded compared to the other treatments. This difference did not persist and after ten days, when all animals were sexually mature, there was no significant size difference between treatments (Figure 4.2.3).

- □ 2 million *D.salina*/animal/day
- \+ 1.5 million *D.salina*/animal/day + 0.25 mg pollard
- ◆ 1 million *D.salina*/animal/day + 0.5 mg pollard
- △ 500,000 *D.salina/animal*/day + 0.75 mg pollard
- x 2 mg pollard

Treatment Codes for Figures 4.2.2 to 4.2.3

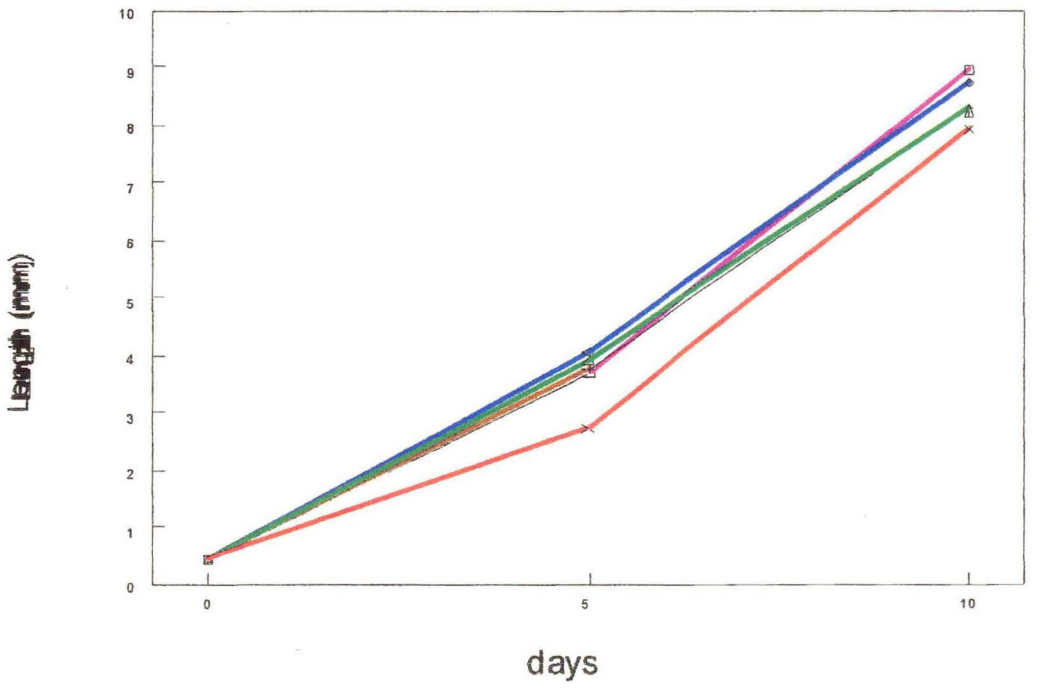


Figure 4.2.1 Growth response of *A.franciscana* to various combinations of *D.viridis* and wheat pollard diet in Experiment 4.5.

Treatment Codes for Figures 4.2.2 to 4.2.3

D.salina*/animal/d + mg wheat pollard/d*

1 = 2 million + 0; 2 = 1.5 million + 0.25 mg

3 = 1 million + 0.5 mg; 4 = 0.5 million + 0.75 mg

5 = 0+1 mg

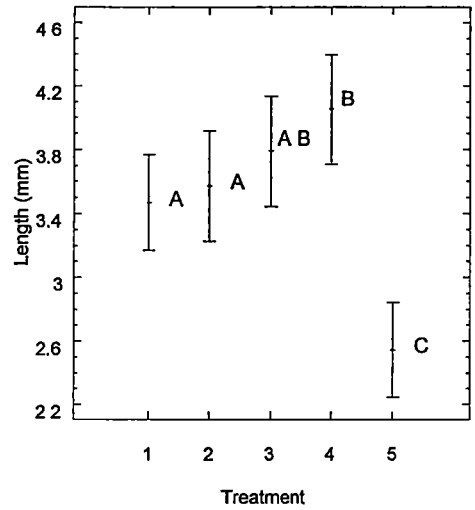


Figure 4.2.2 Means plot (Mean±95% LSD intervals) for *A.franciscana* length after five days feeding in Experiment 4.5. ANOVA p=0.0024. Means sharing a common superscript are not significantly different (p>0.05).

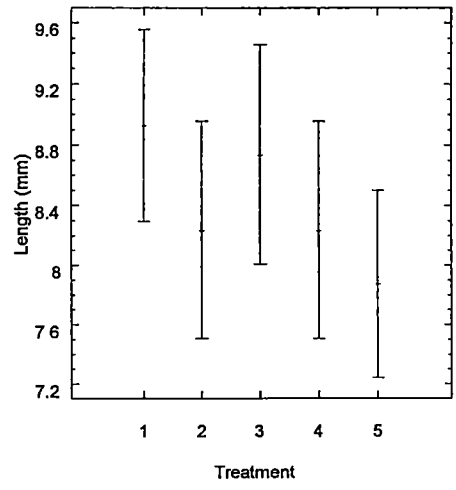


Figure 4.2.3 Means plot (Mean±95% LSD intervals) of *A.franciscana* lengths after ten days in Experiment 4.5. ANOVA p= 0.44.

4.2.3.2 Experiment 4.6: the Growth Efficiency and Population Response of Communal A.franciscana *In 500 mL Beakers to* D.salina *and Mixed D.salina/Wheat Pollard Diet.*

There were highly significant (p= 0.007) differences in animal lengths after eleven days feeding (Figure 4.2.4), with animals in Treatment 1 (just *D.salina)* being smaller than animals in the other treatments which were receiving additional food in the form of wheat pollard. *A.franciscana* populations were not significantly different after eleven days (Figure 4.2.5) and there was no clear pattern of population response. There were, however, great mortalities of nauplii in all cases and the initial populations of 500 nauplii declined unevenly, even among replicates of individual treatments. The final dry weights (Figure 4.2.6) show a clear and highly significant pattern (p=0.0028) of increasing weight with increased food input. The total dry weights are a much truer indication of productivity within beakers and largely eliminate the problems of analysing data including small populations of large animals and large populations of small animals within individual treatments. The mean growth efficiencies are given in Table 4.2.1 and show that growth efficiencies of about 20% were achieved on a diet of *D.salina* alone and between 5% and 6% when additional food in the form of wheat was added. Dry weights were determined on the basis of the length/dry weight relationship presented in Appendix 4.

Table 4.2.1: Mean growth efficiencies in each Treatment in Experiment 4.6

Treatment	Total Weight food/beaker (µg)	Mean total weight (µg) *A.franciscana*/treatment	Growth Efficiency %
Control	8930	1830	20
+30 mg wheat	338930	17946	5.3
+60 mg wheat	668930	40107	6.0

Treatment Codes for figures 4.2.4, 4.2.5 and 4.2.6 (Experiment 4.6).

1. 12.5 million *Dunaliella salina* cells/beaker/day.
2. 12.5 million *D.salina* cells plus 30 mg pollard/beaker/day.
3. 12.3 million *D.salina* cells plus 60 mg pollard/beaker/day.

Means sharing a common superscript are NS (P>0.05)

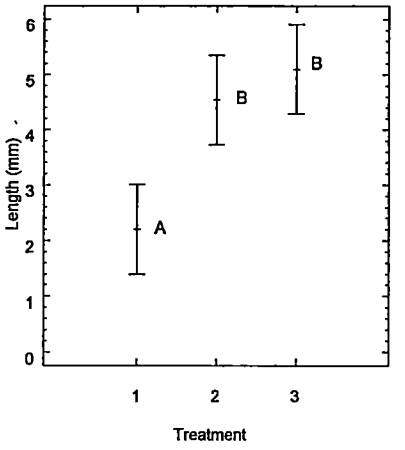


Figure 4.2.4: Means Plot (Mean ±95% LSD intervals) comparing 11 day animal lengths in Experiment 4.6 (ANOVA p=0.0067).

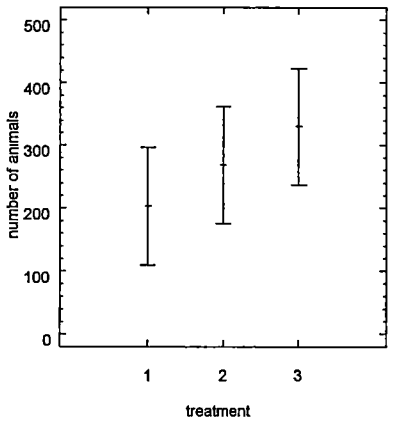


Figure 4.2.5: Means Plot (Mean ±95% LSD intervals) of *A.franciscana* population greater than 5 mm long after 11 days in Experiment 4.6 (ANOVA p=0.35).

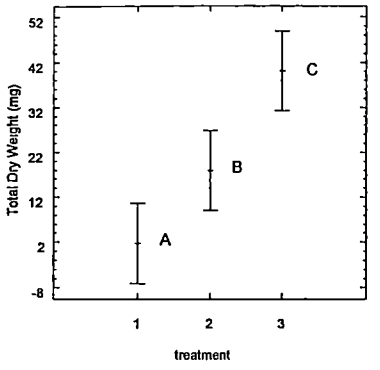


Figure 4.2.6. Means Plot (Mean ±95% LSD intervals) of total dry weights after 11 days in Experiment 4.6 (ANOVA p=0.0028)

4.2.3.3 Experiment 4.7: The Growth Efficiency and Population Response of Communal A.franciscana *In 1,000 L Outdoor Tanks to* D.salina *and Mixed D.salina/Wheat Pollard Diet.*

There were highly significant (p= 0.001) differences between treatments for final wet weights of *A.franciscana* (Figure 4.2.7) and treatments receiving wheat had greater final weights. The *D.salina* culture collapsed in the second week of the trial causing an interruption to supplies of microalgae food to all tanks (Table 4.2.2). During the period required to reestablish this culture the tanks received brine directly from the saltfield which provided water exchange and low levels of various microalgae. The impact of wheat addition on brine pH, density and dissolved Oxygen at 1300 h, when oxygen concentration in the saltfield brines is normally maximal, was tested at the end of the trial. The only significant difference detected between treatments was a highly significant decrease in brine pH with increasing addition of wheat (Figure 4.2.8).

Table 4.2.2: Population of microalgae (cells*10^3/mL) in culture brine flowing into *A.franciscana* tanks in Experiment 4.7.

Time	*D.salina*	*T.suecica*	Other
Initial	90	0	0
Week 1	85	0	0
Week 2	0	0	0.3
Week 3	0	0	0.3
Week 4	0	34.8	0

Note: The plankton pond was empty during Week 2 and 3. At this time the tanks received brine directly from the saltfield containing between 0.2 and 0.3 microflagellatesx10^3/mL. The plankton pond was again operational in Week 4 but *T.suecica* had replaced *D.salina* as the dominant microalgae.

Treatment Codes for Experiment 4.7, Figures 4.2.7 and 4.2.8
1=Microalgae
2=Microalgae+25g pollard/day
3=Microalgae+50g wheat pollard/day

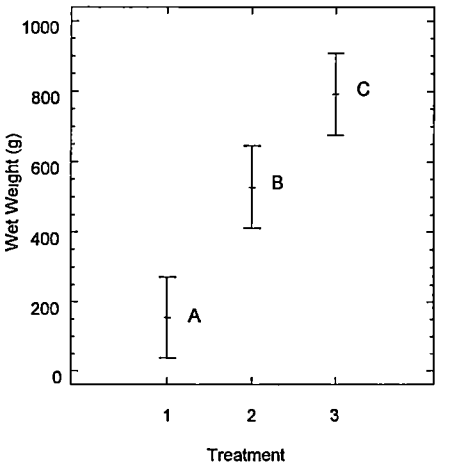


Figure 4.2.7: Means plot (Mean ±95% LSD intervals) for final wet weight (g) per treatment in Experiment 4.7 (ANOVA $p< 0.001$). Means sharing a common superscript are not significantly different.

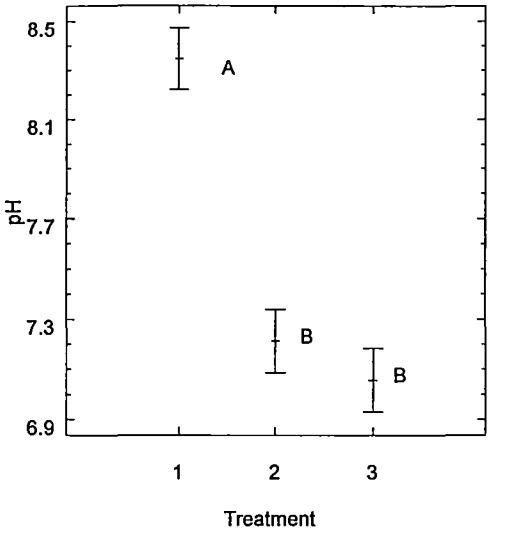


Figure 4.2.8. Means Plot (Mean ± 95% LSD intervals) of brine pH in Experiment 4.7. ANOVA $p<0.001$. Means sharing a common superscript are not significantly different.

4.2.3.4 Experiment 4.8: The growth efficiency and population response of communal A.franciscana *in 1,000L outdoor tanks to wheat pollard with and without* D.salina *supplements.*

There were highly significant differences in final wet weights (g)($p=0.02$) between treatments with Treatment 3 having significantly more biomass than Treatments 1 and 2 (Figure 4.2.9). *A.franciscana* populations were monitored during the trial but results were highly variable within each replicate and were not included.

The population of *D.salina,* in algal tanks paired with the three *A.franciscana* tanks comprising Treatment 3, during the trial is shown in Table 4.2.3. All tanks were contaminated with *A.franciscana* by the final week. The dry weight of each *D.salina* cell has been estimated at $6.5*10^{-11}$g. The total dry weight of cells fed in 100 L/day to each adjacent *A.franciscana* tank would therefore be about 1.3 g per day with a microalgae concentration of about 200,000 cells/ mL.

There was no significant difference between treatments of brine pH at the end of the trial (Figure 4.2.10).

Table 4.2.3: *D.salina* numbers in paired tanks (mean of five counts *10^3/mL) in Experiment 4.8

Tank	Init.	Week 1	Week 2	Week 2 Rpt	Week 3*
1.	26	104	90	88	28
2.	34	166	140	152	14
3.	52	198	270	200	64

* All tanks contaminated with *A.franciscana*.

Note: The dry weight of each *D.salina* cell has been estimated at 6.5*10^{-11}g. (Appendix 1).The total dry weight of cells fed in 100 L/day per day to each adjacent *A.franciscana* tank would therefore be on average about 0.8 g/day.

Treatment Codes for Experiment 4.8, Figures 4.2.9 and 4.2.10
1: 25g pollard/d +No *D.salina* at any time
2: 25g pollard/d +initial *D.salina*
3. 25g pollard/d + 100 L *D.salina* culture/d.

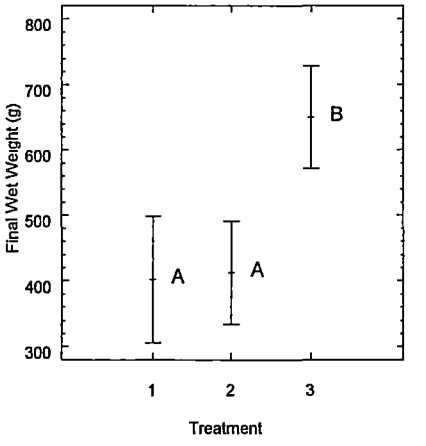


Figure 4.2.9. Means Plot (Mean±95% LSD intervals) for final wet weights (g)/1,000 L in Experiment 4.8. ANOVA p=0.019. Means sharing a common superscript are not significantly different.

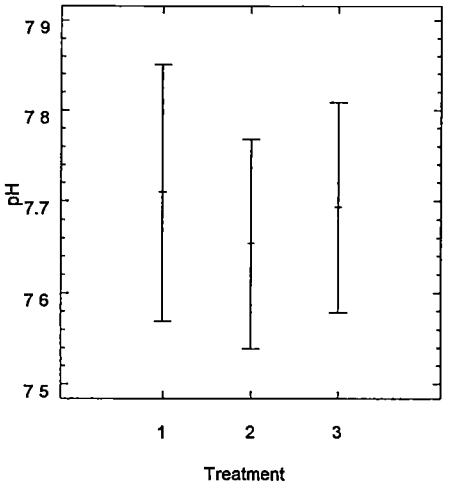


Figure 4.2.10. Means plot of brine pH (Mean±95%LSD intervals) in Experiment 4.8. ANOVA p=0.84.

4.2.4 Discussion

The animals fed two million *D.salina* cells per day in Experiment 4.5 (Treatment 1) were very similar in size to identically fed animals in Experiment 4.1 and 4.2. The initial growth rates of *A.franciscana* fed solely on wheat were retarded compared to the animals with at least some microalgae in their diets (Figure 4.2.1) so that after five days feeding there was a significant difference (p=0.0024) in animal lengths between treatments (Figure 4.2.2). After ten days, when all animals were sexually mature, there was no significant size difference between treatments (Figure 4.2.3). *A.franciscana* fed solely on wheat were, however, very pale and translucent and appeared to lack haemoglobin. *A.franciscana* at even the lowest feeding level for *D.salina* were, however, coloured and virtually indistinguishable from animals fed solely on *D.salina*. Douillet (1987) found that bacteria were essential for the growth of *Artemia* fed inert food. Although the culture brines were changed daily there may well have been some bacteria present, contributing to the *Artemia's* diet even though the wheat pollard was not presoaked.

Experiment 4.6 confirmed that wheat pollard was an effective food for *A.franciscana* with wheat additions resulting in significantly larger animals (p=0.0067) (Figure 4.2.4) although population densities were highly variable and not significantly different. The overall dry weights exhibited highly significant differences (p=0.003) (Figure 4.2.6) and yielded a much clearer picture of animal productivity by

largely eliminating the complication caused by small populations of large animals and large populations of small animals within treatments. The reasons for the variation in populations is not clear as all beakers initially had the same populations. The initially high populations were used to ensure severe competition between animals from Day 1.

The 20% growth efficiency of algae fed animals was much greater than the 5-6% determined for animals with wheat supplements (Table 4.2.1). This is in keeping with the results of Experiment 4.5 which also indicated that *D.salina* was a much more effective food than wheat pollard on a dry weight basis. The experiment was terminated prior to the achievement of full maturity, even at the highest feeding level. It is therefore not possible to determine what adult population would have been finally supported at each feeding level. It is probable that, in such a food limited situation, that the physical size of the *Artemia* in each beaker represents the maximum size achievable with the mean amount of food received in each treatment and that as the animals grow the populations will decline. The mean length of animals in Treatment 1 was 2.1 mm and these received an average of 3.2 µg dry weight of *D.salina* per day (feed input of 25,000 cells/animal/d is very low). This compares to Treatment 3 with the highest feeding level where animals of mean length about 5 mm were being supported by an average of 187 µg of mixed *D.salina* and wheat pollard diet.

Experiment 4.7 was seriously disrupted by an interruption of microalgae food in the second and third weeks of the experiment (Table 4.2.2). During this period animals in tanks receiving only microalgae were receiving low levels of food. This was still, however, sufficient to support a reasonable population of *A.franciscana* amounting to a mean of 155 g wet weight per tank at the end of the trial (Figure 4.2.7). The wet weight of *A.franciscana* in tanks receiving wheat was significantly higher than in tanks without wheat (Table 4.2.1). Despite the fact that the biomass estimates were relatively crude, they confirm the findings of the laboratory Experiments 4.5 and 4.6 that wheat pollard is a suitable food for *A.franciscana* and also confirms its suitability for large scale outdoor culture.

The addition of wheat pollard caused a highly significant decrease in the brine pH (Figure 4.2.8) but other measured brine parameters including Dissolved Oxygen levels in the early afternoon were not significantly different. Substantial deposits of black decaying detritus were present on the bottom of tanks receiving wheat. This material could become a significant problem in pond culture situations. The build up of black detritus was possibly exacerbated by the practice of daily batch feeding. The wheat pollard tends to sink in brine, even in well mixed tanks, and batch feeding results in the loss of some

wheat to the bottom before it can be consumed by the *A.franciscana*. The ideal feeding system would involve continuous input and even distribution of pollard at a rate where most is consumed in the brine column.

In Experiment 4.8 the final wet weights of *A.franciscana* were significantly different (p=0.02) (Figure 4.2.9) with biomass in tanks receiving algae throughout the trial being much greater than in the other two treatments. The addition of *D.salina*, although only a small fraction of the total food input, amounting to 5% on a dry weight basis (Table 4.2.3), had a dramatic difference on final weights. *A.franciscana* weight was double in tanks receiving supplementary *D.salina* although this only accounted for about 5% of the their food on a dry weight basis. The food conversion efficiency (ww *Artemia*/dw food) was close to 1 for tanks receiving supplementary live microalgae and about 0.5 for tanks only receiving pollard. Conversion efficiencies of 1 have been achieved using micronised rice bran in both batch (Bossuyt and Sorgeloos, 1981) and flow through (Brisset et al., 1982) culture without microalgae supplements. There were no significant differences in any of the measured brine parameters, including brine pH, indicating that the difference in weights was not likely to have been influenced by culture conditions. In a review of *Artemia* production in culture tanks produced by Lavens and Sorgeloos (1991) it was concluded that the mass culturing of live microalgae as food for *Artemia* is not economically realistic. The use of a relatively small amount of live microalgae as a supplement to inert feed would, however, greatly influence the economics.

CHAPTER 5: FACTORS AFFECTING THE REPRODUCTIVE RESPONSE OF THE DAMPIER STRAIN OF *A.FRANCISCANA*.

Introduction

Artemia spp. exhibit great flexibility in reproduction. They can reproduce either sexually or parthenogenetically and produce either live young or cysts depending on culture conditions. The ability to switch reproductive mode appears to be related to maternal heterozygosity but the triggering factor has not been conclusively identified (Gajardo and Beardmore 1989). There have, however, been numerous studies into the reproductive mode of *Artemia spp.* encompassing both environmental and genetic factors (Balasundaran et al.,1980; Berthelemy-Okazaki and Hedgecock, 1980; Browne, 1980; Lenz, 1980; Versichele and Sorgeloos, 1980; Vanhaecke and Sorgeloos, 1983; Wear and Haslett, 1987; Gajardo and Beardmore, 1989). An understanding of mechanisms controlling oviparity is fundamental to developing a culture system designed to maximise the production of cysts for commercial purposes.

The brine concentrating ponds at Dampier Salt Limited contain a strain of *A.franciscana* introduced to the saltfield in 1973 from Shark Bay. This is the species used in all experimental work in this report both because of it's practical relevance and because *A.franciscana* has been found to be the most prolific cyst producer in a number of comparative studies (Browne, 1980; Lenz, 1980; Vanhaecke and Sorgeloos, 1983).

The following study was undertaken to determine conditions to maximise the production of cysts from the Dampier strain of *A.franciscana* in large scale intensive culture. This study is presented in five parts:

Part 1. The reproductive response of *A.franciscana* to food supply.

Part 2. The use of screens to minimise competition in communal *A.franciscana* culture.

Part 3. The reproductive response of *A.franciscana* to salinity.

Part 4. The reproductive response of *A.franciscana* to starvation and full sunlight.

Part 5. The reproductive response of *A.franciscana* to Oxygen stress and Iron in large scale outdoor culture.

5.1 The Reproductive and Population Response of Ohe Dampier Strain of A.Franciscana *to Food Supply.*

5.1.1 Introduction

Artemia spp. receiving high levels of food have increased fecundity (Lenz, 1980; Lavens and Sorgeloos, 1987) but starving animals produce better quality cysts than well-fed ones (Lavens and Sorgeloos, 1987). A knowledge of the reproductive response of *A.franciscana* to food quantity and quality is fundamental to optimising feeding systems intended to maximise the production of cysts.

The reproductive response of the Dampier strain of *A.franciscana* to *Dunaliella spp.,* wheat pollard and mixed *Dunaliella*/pollard diet was investigated in a series of experiments which included a study on the effects of pulse feeding. The problem of competition for food was then addressed. No comparable published studies of the effect on reproductive output of *A.franciscana* in relation to different inputs of algae or inert feeds, or pulse feeding in culture systems have been located.

5.1.2 Methods

5.1.2.1 Experiment 5.1: The Reproductive Response of Laboratory Cultured A.franciscana *To D.viridis Mass Cultured in Saltfield Brine.*

A.franciscana grows rapidly on a diet of *D.viridis* with an optimal feeding level of between one to two million cells per animal per day (Chapter 4.1). The optimal feeding level for reproductive output was assessed in the following experiment.

A cohort of Dampier strain *A.franciscana* was reared in the laboratory for three weeks on a diet of *D.viridis.* A pair of sexually mature animals were then transferred to each of twelve labelled 100 mL beakers containing 50 mL brine of density 1.08 g/mL. Four feeding treatments, encompassing the range 500,000 to four million cells/animal/day, were then randomly allotted to the beakers in triplicate.

The population of mass cultured *D.viridis* was determined daily from a mean of five random counts, each encompassing 25 μL on an "Improved Neubauer" haemocytometer. The appropriate volumes of culture were then dispensed with a 5 mL pipettor adjustable in 0.01 mL increments. The animals were transferred daily to fresh brine to which the food had previously been added and the old brine was filtered through GF/C filter papers and retained cysts and nauplii were counted under a dissecting microscope and not returned to the beakers.

5.1.2.2 Experiment 5.2: The Reproductive Response of Laboratory Cultured A.franciscana *to Ground Wheat Pollard and the Effect of Competition and Pulse Feeding on this Response.*

An experiment was undertaken to determine the effect of quantity of finely ground (particle size<50 μm) wheat pollard flour on the reproductive output of *A.franciscana*. Wheat pollard was found to be a good supplementary food for *A.franciscana* in Chapter 4.2 and its value as a food for reproducing animals needed to be assessed. The removal of nauplii on a weekly basis was undertaken with some treatments to prevent the expansion of the population during the course of the experiment. The use of periodic pulses of additional food was also trialed to determine whether this would be an effective method of ensuring high reproductive output whilst preventing the recruitment of nauplii into the adult population. Nauplii are less efficient feeders than adults and should starve first (Schrehardt, 1987).

A.franciscana reared solely on a diet of wheat pollard in 1,000 L outdoor tanks were returned to the laboratory. Ten mating pairs were added to each of 24 labelled 1 L beakers containing 500 mL of brine of density 1.08 g/mL. Five treatments were applied at random in quadruplicate:

Treatment	Wheat/animal	Nauplii Removal
1.	0.5 mg/day	Weekly
2.	1 mg/day	Weekly
3.	2 mg/day	Weekly
4.	4 mg/day	Weekly
5.	1 mg/day	Not removed
6.	0.75 mg/day for 6 days +2.5 mg/once a week	Not removed

Wheat pollard, premixed with brine of density 1.08g/mL was dispensed daily with a 5 mL pipettor adjustable in 0.1 mL increments. The treatments were applied to all beakers for four days before the commencement of the experiment to attune the animals to the treatments. During this time the brine was changed daily. Treatment 4

was abandoned after the first four days because most of the adults had died and the feeding level was obviously too high.

The animals were then transferred to fresh brine and the experiment ran for two weeks without change of brine.

After one week, adult animals in Treatments 1 to 3 were temporarily removed from their culture brine and placed in fresh brine. The culture brines were then filtered through GF/C filter papers which retained nauplii and cysts for counting. The filtered brines and adults were then returned to the beakers. Animals in Treatments 5 and 6 were not handled.

At the conclusion of the experiment, cysts produced in each beaker were adhering to the sides of the culture vessel and were counted in situ. The brines were then screened through a 450 μm screen which retained adult animals and well developed nauplii but allowed cysts, uneaten wheat and faecal material to pass through the screen. The animals were rinsed from the screen with 100 mL of microfiltered brine of density 1.08 g/mL into a clean beaker. This brine was then filtered through a GF/C filter paper and the animals counted.

5.1.2.3 Experiment 5.3: The Effect of Pulse Feeding and Animal Harvesting on A.franciscana *Populations and Their Production of Cysts in 1,000 L Tanks.*

A follow up experiment to Experiment 5.2 was designed in order to determine the effect of pulse feeding on *A.franciscana* reproductive output and populations in 1,000 L tanks. A second treatment was applied where a portion of animals were harvested each week in an effort to reduce competition and possibly stimulate reproductive output from remaining adults.

Twelve by 1,000 L fibreglass tanks were filled with brine of density 1.10 g/mL containing about 100,000 *D.salina* cells per mL from a large plankton pond. *A.franciscana* were netted from a pond and about 30,000 animals were added to each of the twelve tanks which were aerated with an axial air blower delivering air through perforated PVC pipes. The additions of animals were undertaken by adding approximately similar wet volumes of animals to each tank; thoroughly mixing the culture brines within the tanks and; determining the population in 1 L samples. Additional animals were then added to tanks with low populations until all tanks had approximately 30 animals /L. Fresh water from an adjacent tank was added to all tanks weekly to replace evaporative losses. Three treatments were applied at random and in quadruplicate to the tanks. Wheat pollard flour was homogenised in water and carefully measured aliquots were then added to the tanks daily for feeding which in all cases averaged 20 g of

flour per tank per day. The initial feeding rate/animal was higher than in Experiment 5.2 but populations were expected to expand during the course of the experiment.

Treatment	Feeding (wheat pollard)	Harvest
1. Pulse feeding	15 g/day for 6 days and 50g on Day 7	Nil
2. 20% harvest	20 g/day	20% (1 mm screen)
3. Control	20 g/day	Nil

The population of animals was monitored weekly by filtering the contents of multiple samples from each tank through filter papers. Initial counts (3*500 mL samples), weekly counts (mean of three by 100 mL samples) and final counts after five weeks (mean of six by 100 mL samples) were undertaken. Brines were homogenised with an oar prior to and between samples. A count of cysts in all collected brines was undertaken on the final day.

A wet weight of the 20% of harvested animals in Treatment 2 was obtained each week and on the final day the total contents of each tank were collected on a screen and weighed.

Weekly pH and brine density readings were taken.

5.1.2.4 Experiment 5.4: The Reproductive Response of A.franciscana *in 1,000 L Outdoor Culture to Pulse Feeding with* D.Salina.

In Experiment 5.2 there was a trend (not significant, p=0.08) towards higher reproductive output for *Artemia* that were pulse fed in laboratory beakers. The follow up Experiment 5.3 did not confirm this for 1,000 L outdoor culture with *A.franciscana* fed on wheat pollard. The reason for this may be related to secondary production from bacterial biomass supported by decomposing wheat pollard and the high feeding rate.

Observations on *A.franciscana* in 0.1 ha pilot ponds and in the salt field have indicated that high reproductive output does occur when abundant microalgae are made available to underfed *A.franciscana*. This observation is consistent with the results of Experiment 5.2. An experiment was designed to determine the reproductive response of *A.franciscana* to pulses of *D.salina* in large scale outdoor culture. These data are fundamental to the development of optimal feeding strategies for cyst producing *A.franciscana*.

The experimental system was similar to that employed in Experiment 5.3. Eight by 1,000 L tanks, equipped with bottom drains which in turn were equipped with air-curtained 150μm screens, were filled with *D.salina* culture containing about two million cells per mL in brine of density 1.10 g/mL. The culture brine was aerated from an axial air blower delivering air through drilled and weighted PVC pipes. 150 g (wet weight) of *A.franciscana* were collected from an experimental pond with a 1 mm mesh net and placed in each of the tanks.

1. Two treatments were applied to the tanks at random as follows.
2. L daily replacement with *D.salina* culture brine.
3. L weekly replacement with *D.salina* culture brine.

The following variables were monitored initially and at the end of the experiment (after three weeks just prior to the next weekly water exchange) using the techniques described in Chapter 2 and Experiments 5.1-5.4. Reproductive output per 20 females was determined by carefully transferring 20 females from each tank into 500 mL of clean culture brine with a pipette. Extreme care was taken to avoid the inadvertent transfer of nauplii or cysts with the females. The females were left for three days (previously determined brood interval from both unreported experiments and Experiment 5.1) before being carefully removed and returned to their original tanks. Spawned cysts and nauplii were collected on gridded GF/C filter paper and counted under a dissecting microscope. The following variables were assessed.

1. Population of animals/L.
2. Population of mature females/L.
3. Reproductive output/ 20 females.
4. Brine pH and density (g/mL).
5. Concentration of *D.salina*/mL*10^3 in tanks.
6. Concentration of *D.salina*/mL*10^3 in plankton pond. (done weekly)

5.1.2.5 Experiment 5.5: Repeat of Experiment 5.4 with Reduced Feeding Frequencies Aad 50% of Total Food Input.

Experiment 5.5 was designed after the completion of Experiment 5.4 when it was apparent that both food input levels and frequency of feeding needed to be reduced greatly. The experiment was similar in design to Experiment 5.4 with the following modifications.

Treatment 1 received 100 L of *D.salina* every two days rather than daily.

Treatment 2 received 700 L of *D.salina* culture brine each fortnight rather than weekly.

The experiment ran for 17 days with final reproductive output data collected from animals three days after the final Treatment 2 feeding.

5.1.3 Results

5.1.3.1 Experiment 5.1: The Reproductive Response Of Laboratory Cultured A.franciscana *To* D.viridis *Mass Cultured In Saltfield Brine.*

The total reproductive output per beaker in the first and second weeks is shown in Table 5.1.1. Much of the reproductive output in the first few days of the experiment can be assumed to be from animals that were pregnant prior to the commencement of the experiment. In the second week there were mortalities in Treatments 1 and 2 which were receiving low volumes of food and reproductive output in these treatments was very low. Cyst production was low and sporadic throughout the trial and not statistically meaningful but spawnings did occur about every three days consistent with a number of previous (not reported) experiments.

Table 5.1.1: Total reproductive output per surviving female *Artemia* during first (No mortalities) and second weeks of Experiment 5.1

D.viridis cellsx10^6/ *Artemia*/d	Week 1			Week 2		
	Mean	SE	n	Mean	SE	n
1. 500,000	89.7	7.5	3	0	0	1
2. 1x10^6	82.7	24	3	4	4	2
3. 2 x 10^6	78.7	5.5	3	20.7	2.4	3
4. 4 x 10^6	104	6.9	3	33.1	8.1	3

ANOVA shows no significant difference ($p>0.05$) between treatments in Week 1 ($p = 0.32$) and in Week 2 ($p = 0.067$) although mortalities were high in low feed rate treatments.

5.1.3.2 Experiment 5.2: The Reproductive Response of Laboratory Cultured A.franciscana *to Ground Wheat Pollard and the Effect of Competition And Pulse Feeding on this Response.*

There were highly significant differences in total reproductive output, cyst production and final adult populations with level of wheat pollard per animal per day (Table 5.1.2). A feeding level of 1 mg was optimal with both highest reproductive output and greatest survival of adults. The lower 0.5 mg feeding level appears too low and 2 mg per animal per day too high. The 4 mg per animal per day treatment was obviously far too high (data not included) and resulted in the death of most of the experimental animals.

Pulse feeding at the 1 mg per animal per day level did not significantly affect either total reproductive output or final adult populations (Table 5.1.3). However, there was a trend towards high cyst output with pulse feeding (p=0.08).The removal of offspring did not significantly affect the adult population but did significantly increase reproductive output compared to the treatment where offspring were retained at the 1 mg per animal per day feeding level (Table 5.1.4).

Table 5.1.2: Comparison of reproductive output and final adult populations at three daily feeding levels of wheat pollard in Experiment 5.2.

Treatment	Total Offspring			Total cysts			Final adult pop.		
	Mean	SE	n	Mean	SE	n	Mean	SE	n
1. 0.5 mg	169[a]	50	4	167.5[a]	49.8	4	14.5[a]	0.5	4
2. 1 mg	506.3[b]	74.4	4	497.8[b]	74.27	4	19[b]	0.9	4
3. 2 mg	188.3[a]	23.8	4	144.3[a]	22.8	4	17.3[b]	1.3	4
Probability	0.003			0.002			0.024		

Means sharing a common superscript are not significantly different (p>0.05).

Table 5.1.3: Comparison of reproductive output and final populations between evenly fed and pulse fed treatments receiving a mean of 1 mg pollard/animal/day in Experiment 5.2.

	Total Offspring			Total cysts			Final adult pop.		
Treatment	**Mean**	**SE**	**n**	**Mean**	**SE**	**n**	**Mean**	**SE**	**n**
5. Even	258.8	29.8	4	204.5	25.5	4	21.3	0.95	4
6: Pulse	419	88.2	4	399.8	883	4	22	0.7	4
Probability	0.14			0.08			0.55		

Table 5.1.4: Comparison of reproductive output and final animal populations between the treatment where offspring were removed and where offspring were retained at a feeding level of 1 mg pollard/animal/day in Experiment 5.2.

Treat	Total Offspring			Total cysts			Final adult pop.		
	Mean	**SE**	**n**	**Mean**	**SE**	**n**	**Mean**	**SE**	**n**
1. Removed	506.3	74.4	4	497.8	74.3	4	19	0.91	4
2. Retained	258.8	29.8	4	204.5	25.2	4	21.3	0.95	4
Probability	0.022			<0.001			0.14		

5.1.3.3 Experiment 5.3: The Effect Of Pulse Feeding and Animal Harvesting on A.franciscana *Populations and Their Production of Cysts in 1,000 L Tanks.*

There were no significant differences between treatments in initial *A.franciscana* populations, final *A.franciscana* populations, and final total wet weight of animals (Table 5.1.5).

There was no significant difference in the final number of cysts/L between treatments (Table 5.1.6).

There was no significant difference in the wet weight of animals harvested from filtering 20% of the culture brine in Treatment two in the second or fourth week of the experiment (Table 5.1.7).

There was no significant difference in brine pH or brine density at the end of the trial (Table 5.1.8).

Table 5.1.5: Initial and final populations of *A.franciscana* /L and final wet weight in Experiment 5.3.

Treat	Init.Population/L			Final Pop./L			Final wet weight g		
	Mean	SE	n	Mean	SE	n	Mean	SE	n
1. Pulse	29.5	2.2	4	115.5	34.2	4	613.7	50.4	3
2. Harvest	32.5	3.3	4	99.75	8.51	4	649.8	73.4	4
3. Control	33.5	2.2	4	113.75	16.42	4	714	46.4	4
Probability	0.56			0.86			0.53		

Table 5.1.6: Final numbers of cysts/L in Experiment 5.3.*

Treatment	Mean	Std. Error	n
1. Pulse fed	54.75	18.47	4
2. 20% Harvest	55.0	8.36	4
3. Control	55.75	8.49	4
Probability	0.998		

*Foaming of tanks can lead to cyst loss.

Table 5.1.7: Comparison of wet weights harvested in second and fourth weeks of Experiment 5.3.

Week	Mean (g)	Std. Error	n
2	53.22	5.94	4
4	50.76	6.7	4
Probability	0.78		

Table 5.1.8: Comparison of Final Brine pH and Density in Experiment 5.3.

Treatment	pH			Brine density		
	Mean	SE	n	Mean	SE	n
1. Pulse	7.76	0.067	4	1.1184	0.0015	4
2. Harvest	7.77	0.03	4	1.1164	0.0005	4
3. Control	7.8	0.034	4	1.1160	0.0004	4
Probability	0.78			0.20		

5.1.3.4 Experiment 5.4: The Reproductive Response Of A.franciscana *in 1,000 L Outdoor Culture to Pulse Feeding of* D.salina.

The total populations of *A.franciscana*/L at the beginning and end of the experiment are shown in Table 5.1.9 and the treatment means were not significantly different. The populations of mature females are shown in Table 5.1.10 and were not significantly different at the beginning or end of the experiment. The reproductive output per 20 females was also not significantly different. The population of *D.salina*/mL at the end of the experiment is shown in Table 5.1.11 and although there was no significant difference between treatments the populations were very high and in fact exceeded the population of *D.salina*/mL in the pilot pond used to feed the tanks. This indicates that after three weeks feeding, the *A.franciscana* population was not sufficiently high to completely strip the *D.salina* or even to keep pace with microalgal production within the tanks. Brine density (g/mL) was significantly higher in the 700 L/week treatment which had been concentrating for one week with no water exchange.

Table 5.1.9: Comparison of *A.franciscana* populations initially and after three weeks in Experiment 5.4.

Treatment	Initial population/L			Final Population/L		
	Mean	SE	n	Mean	SE	n
1. 100 L/day	25.7	1.81	4	36.6	5.9	4
2. 700 L/week	21	1.56	4	25.6	2.74	4
Probability	0.096			0.14		

Table 5.1.10: Comparison of female populations initially and after three weeks and final reproductive output/20 females in Experiment 5.4.

Treatment	Initial Population/L			Reprod.Output/20 Females.			Final Population/L		
	Mean	SE	N	Mean	SE	N	Mean	SE	N
1. 100 L/d	9.95	1.05	4	529	197	4	2.9	0.67	4
2. 700 L/week	8.9	1.09	4	204	26	3*	3.9	0.17	4
Probability	0.52			0.22			0.18		

* One replicate spilt in processing and not included.

Table 5.1.11: Comparison of final *D.salina*/mL and brine density(g/mL) in Experiment 5.4.

Treatment	*D.salina*/mL			Brine density (g/mL)		
	Mean	SE	n	Mean	SE	n
1. 100 L/day	80.5	16.25	4	1.1571	0.0019	4
2. 700 L/week	268.2	180	4	1.1632	0.0006	4
Probability	0.34			0.02		

Note: Final concentration of *D.salina* in plankton pond = 736/mL.

5.1.3.5 Experiment 5.5: Repeat of Experiment 5.4 with Reduced Feeding Frequencies and 50% of Total Food Input.

The initial total populations of *A.franciscana* were significantly different between treatments due to the presence of many immature animals in some tanks (Table 5.1.12). The adult population, as reflected in the population of mature females, was not initially significantly different (Table 5.1.13). There was no significant difference between treatments in either total population size, or population of mature females after 2.5 weeks (Tables 5.1.12 and 5.1.13). Reproductive output between treatments was not significantly different at either the beginning or end of the experiment (Table 5.1.14) and animals in all tanks were primarily oviparous throughout the experiment (Table 5.1.15). The brine density (g/mL) of the plankton pond was high in the final week of the experiment resulting in significantly higher brine densities in tanks

receiving 100 L turnover of brine every two days (Table 5.1.16). The pH of the high density culture brines was lower in keeping with normal brine chemistry processes (Table 5.1.16).

Table 5.1.12: Comparison of *A.franciscana* populations initially and after 2.5 weeks in Experiment 5.5.

Treatment	Initial population/L			Final Population/L		
	Mean	SE	n	Mean	SE	n
1. 100 L/2 days	3.4	0.39	4	24.9	4.17	4
2. 700 L/2 weeks	5.84	0.74	4	20.35	2.52	4
Probability	0.027			0.4		

Table 5.1.13: Comparison of female populations initially and after 2.5 weeks in Experiment 5.5.

Treatment	Initial population/L			Final population/L		
	Mean	SE	n	Mean	SE	n
1. 100 L/2 days	0.6	0.12	4	6.8	1.4	4
2. 700 L/2 weeks	1.45	0.54	4	4.9	0.55	4
Probability	0.18			0.25		

Table 5.1.14: Comparison of initial and final reproductive output/20 females in Experiment 5.5.

Treatment	Initial Offspring/10F			Final Offspring/20F		
	Mean	SE	n	Mean	SE	n
1. 100 L/2 days	189.8	52.5	4	378	14.8	4
2. 700 L/2 weeks	151.3	21.2	4	328	43.7	4
Probability	0.53			0.32		

Table 5.1.15: Comparison of initial and final Cyst/total offspring *100 in Experiment 5.5.

Treatment	Initial			Final		
	Mean	SE	n	Mean	SE	n
2. 100 L/2 days	99.43	0.58	4	98.75	0.66	4
1. 700 L/2 weeks	94.9	1.85	4	92.53	3.97	4
Probability	0.06			0.17		

Table 5.1.16: Comparison of brine pH and density(g/mL) in Experiment 5.5.

Treatment	pH			Brine density (g/mL)		
	Mean	SE	n	Mean	SE	n
1. 100 L/day	7.58	0.009	4	1.1438	0.0019	4
2. 700 L/week	7.76	0.01	4	1.1172	0.0013	4
Probability	< 0.001			<0.001		

Note: Final concentration of *D.salina* in plankton pond = 332,000 ± 28,480 cells/mL (n = 5). Initial population of *D.salina* was about 2,000,000 cells/mL.

5.1.4 Discussion

In Experiment 5.1 batches of offspring occurred every three days during the first week of the trial at all feeding levels suggesting a three-day brood interval for Dampier *A.franciscana* in the laboratory. This was a relatively consistent pattern for all laboratory cyst producing experiments and is consistent with the relatively short brood interval (3.5 to 4.8 days) reported for Old World bisexual *Artemia* (i.e *A.franciscana*) (Lenz, 1987). Browne et al. (1984) cited in Lenz and Browne (1991) in a comparative study of 12 *Artemia* populations encompassing four species found distinct reproductive and life-span characteristics with *A.franciscana* having the longest reproductive period, most offspring per brood and highest percent of cysts hatched, particularly when brine temperatures exceeded 25°C.

Much of the first weeks reproduction occurred in the first few days and it is probable that these animals were pregnant at the start of the trial. This would confound the data. During the second week there were mortalities

at the lowest feeding levels, possibly due to starvation, and reproductive output at the low feeding levels was very low. This suggests that feeding levels of one million *D.viridis* cells/animal/day may be too low to support reproductive output and that a minimum of two million *D.viridis*/day is needed for each animal. This is the upper range for the optimum feeding level for growth found in Chapter 4.1. Cyst production was poor in all treatments and produced cysts were soft (easily squashed with a needle) and grey (the shell did not appear to have formed properly). Attempts to hatch these cysts were not successful.

The second experiment (5.2) showed that the optimum quantity of wheat pollard for both total reproductive growth and animal survival was 2 mg per animal per day (Table 5.1.2). Higher feeding levels retarded growth in the 500 mL unaerated beakers in the laboratory.

The effects of pulse feeding and offspring removal on reproductive output were conducted at the suboptimal feeding level of 1 mg per animal per day. This was not known to be suboptimal when the experiment was designed although it was known that 1 mg of wheat pollard per animal per day was sufficient to give good growth rates (Chapter 4.2). Despite this, it appears that the removal of offspring and the subsequent decrease in competition for food results in highly significant increased fecundity (Table 5.1.4). This is consistent with the findings of Experiment 5.1 and studies on the reproductive response of *Artemia* in a number of natural systems where reproductive output correlates significantly with food level (Lenz and Browne, 1991)

There were some indications that pulse feeding has the potential to increase reproductive output even though it did not do this significantly at the suboptimal 1 mg pollard per animal per day feeding level (Table 5.1.3). This aspect was pursued in subsequent experiments.

In Experiment 5.3 neither animal harvesting or pulse feeding was effective in limiting the mean population of *A.franciscana* in 1,000 L tanks (99.8-115.5 animals/L) at the end of the trial (Table 5.1.5). There was also no significant difference in the total wet weight of animals harvested from the tanks at the end of the experiment indicating that the animals in different treatments were of similar size (Table 5.1.5). There was a small amount of uneaten wheat and detritus in the tanks at the end of the trial which may have increased the wet weight results. If reproductive output is strongly influenced by food supply, as indicated in the earlier experiments, then reproductive output per treatment should also have been very similar and this is indicated in Table 5.1.6 where mean cyst numbers/L between treatments were almost identical at 55 cysts/L (p value close to 1). This does not mean there were no cyst losses from the tanks which had a tendency to foam and cannot be used as a guide to reproductive output.

No attempts at increasing reproductive output via a reduction in intraspecific competition seem to have been reported in the literature. The weekly removal of 20% of the population >1 mm on a weekly basis was

sustainable, as indicated by the final populations and wet weights (Table 5.1.5), and the fact that there was no significant decrease in wet weight harvest from the second to the fourth week (Table 5.1.7). If this weight equates to the maximum achievable standing crop of *Artemia* at the 20 g/day/1,000 L pollard feeding level then it is probable that conditions of high competition prevailed for much of the time. Weekly harvesting appears to be an ineffective method of increasing *A.franciscana* fecundity with cyst production very similar to the control tanks (Table 5.1.6).

Pulse feeding in Experiment 5.3 was also not effective in limiting population growth and stimulating cyst production. A high level of secondary nutrition from bacteria feeding on uneaten wheat and faecal material is likely because this material was not removed from the tanks. This is a well documented phenonemum and in fact is essential for the nutrition of *Artemia spp.* (Douillet, 1987). If this is occurring it would affect the effectiveness of pulse feeding by supplying a high level of nutrition at all times. This problem would be minimised by the use of microalgae food alone as undertaken in Experiment 5.4.

Experiment 5.4 was designed to test the reproductive stimulus of pulses of food fed to starving animals. The high productivity of *D.salina* within the tanks, however, ensured animals in both treatments were at all times well fed. It is not surprising, therefore, that no significant differences in animal populations or reproductive output were found.

Experiment 5.4 was repeated in Experiment 5.5 with half the brine turnover. The *A.franciscana* in this experiment effectively cleared the brine in all tanks but feeding strategy had no significant effect on animal population size (Tables 5.1.12 and 5.1.13); reproductive output (Table 5.1.14) or; percent oviparity (Table 5.1.15) with animals remaining basically in oviparous mode throughout the experiment. The experiment was confounded in the final week by a collapse of the microalgae food supply from an initial 2,000,000 cells per mL down to about 300,000 cells per mL at the end of the experiment. The collapse in food supply occurred at the end of the experiment when the microalgae pond used as a food supply for experimental animals became contaminated with *A.franciscana*.

5.2: The Use Of Screens To Remove Offspring and Minimise Competition in Communal A.franciscana Culture

5.2.1 Introduction

Food level was a critical factor in reproductive output (Experiment 5.2). An effective technique for minimising *A.franciscana* population growth and reducing the competition between animals in communal culture has not been developed although preliminary trials revealed that selective removal of offspring was promising (Treatment 2, Experiment 5.2).

The following series of experiments were conducted to develop selective screening techniques to minimise *A.franciscana* population growth and to determine the reproductive response. Flow-through culture systems using a variety of self cleaning screens have been well researched for the production of biomass (Lavens and Sorgeloos, 1991). This information was the starting point in developing the various screening techniques used in the following experiments.

5.2.2 Methods

5.2.2.1 Experiment 5.6: The Use Of 500 μ m and 700 μ m Screens to Limit A.franciscana Population Growth in 1,000 L Outdoor Tanks.

This experiment was undertaken after earlier strategies employed to limit the population growth of *Artemia* in 1,000 L tanks were not successful (Section 5.1, Experiment 5.3). This included pulse feeding and the partial harvest of all animals on a periodic basis.

Newly hatched nauplii are about 400 μm long. A screen size of 500 μm should allow the passage of newly hatched nauplii and a 700 μm screen larger nauplii.

Nine by 1,000 L fibreglass tanks were filled with brine of density 1.08 g/mL which was aerated via perforated PVC pipe fed from an axial air blower. About 10,000 adult *A.franciscana* were netted from the saltfield ponds and added to each tank to give an initial population of about 10 animals/L. Small submersible pumps delivering about two L of brine/minute were placed within primary screens (500 μm or 700 μm) within the tanks with effluent screened by a 300 μm mesh secondary screen, to capture any offspring passing through the primary screens. The effluent was then returned to the tank. Three treatments were applied at random to the nine tanks as follows.

1. Control (no screening).
2. Primary screen 500 μm
3. Primary screen 700 μm

Each tank received 50 g of finely ground wheat pollard per day which on the basis of previous experiments should support about 50 animals/L and therefore stimulate high reproductive output in all tanks initially containing only ten animals/L. The population of *A.franciscana* was monitored initially and at the end of the trial by thoroughly mixing the brines with an oar and collecting three 100 mL samples. The brines were returned to the laboratory and filtered through gridded filter papers before undertaking counts and measurements under a dissecting microscope.

Reproductive output at the end of the trial was monitored by placing five fecund females from each tank in filtered brine and recording offspring spawned.

5.2.2.2 Experiment 5.7: The Use of 0.4 mm and 2 mm Mesh to Limit the Population Expansion of A.franciscana *in 1,000 L Outdoor Tanks.*

The failure of the 0.5 mm and 0.7 mm screens used in Experiment 5.6 to prevent *A.franciscana* population growth can be partially attributed to the clogging of the primary screens. It also appeared likely that a much larger screen size was needed to curb population growth.

This experiment was designed to test the effect of a relatively large 2 mm primary overflow screen on *A.franciscana* population growth and fecundity. Experiment 5.7 was conducted along similar lines to Experiment 5.6 but with some major differences.

1. Screened saltfield brine of density 1.07 g/mL and free of *Artemia* was pumped through each tank at a rate of 2 L/min for eight hours each day. The tanks were equipped with either 0.4 mm (to retain all offspring) or 2 mm (to allow all offspring and even sub-adults to pass through) screens.
2. *Artemia* populations in each tank were monitored by first mixing the brines with an oar. A 1 L wide mouth jar was then used to collect brine from the tank. This was poured through a 1 mm sieve which retained all adult and most sub-adult *Artemia* and a total count made of all animals and fecund females which had conspicuous ovisacs. The process was repeated 5 times for each tank and increased by an order of magnitude the volume screened in Experiment 5.6.
3. Reproductive output from ten fecund females from each tank was monitored at the end of the trial by allowing them to spawn in beakers of filtered brine in the laboratory over a three day period.

5.2.2.3 Experiment 5.8: The Use of 0.4 mm and 1.6 Mm Screens to Restrict A.franciscana *Population Expansion in 1,000 L Outdoor Tanks.*

A third screening experiment was conducted over one week using identical methods to Experiment 5.7 but with a slightly smaller, 1.6 mm, screen size to minimise losses of large sub-adults.

5.2.2.4 Experiment 5.9: The Use Of 0.4 mm and 1.6 mm Screens With and Without Brine Recycling on A.franciscana *Population Growth and Reproductive Output in 1,000 L Outdoor Tanks.*

Previous experiments indicated that a screen size of 1.6 mm through which brine overflows from a pond is sufficient to retain most of the adult *A.franciscana* whilst allowing juveniles to pass through. This in turn prevents population expansion and results in a higher feeding level per adult animal. This in turn may stimulate reproductive output. The main problem with this system is a loss of food through the overflow.

In an effort to confirm initial findings on the effectiveness of overflow screens as a means of restricting *A.franciscana* populations and to gauge the effect of food loss from the system the following experiment was undertaken.

Twelve by 1,000 L fibreglass tanks were filled with field brine of density 1.07 g/mL. The brine was sterilised with 100 ppm chlorine, added as Sodium Hypochlorite, and aerated via perforated PVC pipes fed from an axial air blower. The pools were fertilised with 0.01 g "Aquasol"/L and inoculated with *D.salina* two days after chlorination when no chlorine was detected. The brines became green within a week and were inoculated with 100 g wet weight of mainly adult *A.franciscana* captured from the saltfield with a 1 mm mesh size net. The population of *A.franciscana* in each tank was measured by the following method. The brines were vigorously agitated with an oar for ten sweeps to homogenise the brine. A 1 L sample of brine was then collected and poured through a 1 mm screen. A count of all retained animals, and fecund females (50% of adult population), on the screen was then undertaken. This was repeated five times for each tank. A small aquarium net was then used to transfer animals from one tank to another until the populations in each tank were similar. Four treatments were then applied to the tanks in triplicate as follows:

1. Control tanks (no screening).
2. Recycling of brine through 1.6 mm and 400 μm screens.
3. Overflow through 400 μm screen.
4. Overflow through 1.6 mm screen.

Brine recycling was achieved by placing small submersible pumps within 1.6 mm mesh size screen cages kept clear with air curtains fed from a compressor pump. Brine passing through these pumps was then screened through large 400 μm secondary screens to avoid the recycling of animals passing through the 1.6 mm screens. The control tanks are a control to the recycled tanks with no loss of food via overflow.

The overflow tanks were fed from screened pond brine delivered by an overhead manifold into each tank. Overflow screens were kept clear with air curtains but there was a tendency for brine levels to rise and some overflow from at least one tank did occur. The amount of flow through the recycling tanks and the overflow tanks was controlled to give a daily throughput (continuous) of about 500 L (50% of the tank volumes). 50 g of wheat pollard was added to each tank at the end of the pumping period in order to minimise food loss through the overflow system.

5.2.2.5 Experiment 5.10: The Reproductive Response of A. franciscana *To Selective Removal of Offspring Via Overflow Screens.*

The experiment was designed to compare population sizes and reproductive output per female from cultures where offspring, but not adults, were allowed to overflow through a 1 mm screen and cultures where all offspring and adults were retained with a 15 μm screen. (A screen size of 1 mm was found to be necessary to retain adults in bottom draining tanks even though a previous experiment (Experiment 5.7) showed that a 1.6 mm screen was adequate in retaining adult *A.franciscana* in surface draining tanks where water pressures were much lower. A 150 μm screen retains cysts which have a diameter of about 200 μm and nauplii with an initial length of 400 μm.)

The removal of offspring via overflow screens was found to be an effective method of restricting *A.franciscana* population growth in Experiments 5.6-5.9. This was done to stimulate reproductive output by ensuring mature females received abundant food. Reproductive data from these experiments were, however, highly variable. A repeat experiment with a high degree of replication and more intensive monitoring of reproductive output was needed.

There were four randomised replicates for each treatment conducted in 1,000 L outdoor aerated tanks. The tanks were bottom drained and equipped with internal air curtained screens of appropriate mesh size and a valve. The tanks were initially filled with *D.salina* culture from an adjacent 800,000 L pond and 150 g wet weight of *Artemia,* netted with a 1 mm mesh sized net from another adjacent pond was placed in each tank. Each day 500 L of brine was drained from each tank through the screens. The tanks were then filled with 500 L of *D.salina* culture brine from the microalgae pond and 50 g of wheat pollard previously homogenised in 1 L of culture brine was added to each tank. Screened brines (150 μm secondary screens) were checked regularly for offspring. Brine pH and density were monitored weekly and temperature range in one tank over the course of the experiment. Dissolved Oxygen levels were monitored on the final day. The population of adult and sub-adult *A.franciscana* was monitored weekly

by first mixing the tank with an oar and then pouring five consecutive 1 L samples through a 1 mm screen on which screened animals could be counted. Counts of total animals and females with egg sacs were recorded. Reproductive output was monitored by capturing twenty mature females from each tank on a weekly basis and allowing them to spawn in filtered brine over a three day period. On the final day three batches of ten females per tank were used. The brines were filtered through gridded filter papers. Retained offspring were counted under a dissecting microscope on X10 power. At the completion of the experiment a total wet weight of animals retained by a 1 mm screen was recorded for each tank.

5.2.3 Results

5.2.3.1 Experiment 5.6: The Use of 500 μm And 700 μm Screens to Limit A.franciscana *Population Growth in 1,000L Outdoor Tanks.*

The mean initial and final lengths of *A.franciscana* are shown in Table 5.2.1. There was a large increase in *Artemia* population during the trial but no significant differences between treatments after two weeks.

Table 5.2.1: Initial and final *A.franciscana* populations/L in Experiment 5.6

Treatment	Initial			Final (2 week)		
	Mean	SE	n	Mean	SE	n
Control	12.5	1.94	3	32.2	7.17	3
500μm	11.5	1.62	3	29.2	2.83	3
700μm	9.35	0.64	2	39.1	5.89	2
Probability	0.5			0.54		

5.2.3.2 Experiment 5.7: The Use Of 0.4 mm And 2 mm Mesh to Limit the Population Expansion of A.franciscana *in 1,000 L Outdoor Tanks.*

The 2 Way ANOVA table of *A.franciscana* retained by a 1 mm screen (Table 5.2.2) shows there was a significant decline in population due to screen size and a significant overall reduction in population over the one week trial and a significant interaction between these factors. Fecund female (those with ovisacs) population on the other hand did

not change significantly (Table 5.2.3) indicating that the females were too large to pass through the 2 mm screens. Figure 5.2.1 presents the mean and standard error data for all combinations of screen size and time graphically.

Table 5.2.2: 2 Way ANOVA of *A.franciscana* retained by 1 mm screen in Experiment 5.7.

		Mean	SE	n	Probability *
A. Screen Size	0.4 mm	51.3	4.5	6	0.008
	2 mm	318	4.5	6	
B. Time	Initial	54.6	4.5	6	0.003
	Final (1 week)	28.5	4.5	6	
Interaction AB					0.005

*ANOVA p calculated with transformed (√) data means and SE on untransformed data.

Table 5.2.3: 2 Way ANOVA of fecund females retained by 1 mm screen in Experiment 5.7.

		Mean	SE	n	Probability *
A. Screen Size	0.4 mm	7.33	0.79	6	0.08
	2 mm	4.62	0.79	6	
B. Time	Initial	5.87	0.79	6	0.75
	Final (1 week)	6.08	0.79	6	
Interaction AB					0.18

*ANOVA p calculated with transformed (Ö) data means and SE on untransformed data. SE values based on mean square error.

Table 5.2.4: Reproductive output from ten females at end of Experiment 5.7

	Cysts		Nauplii		Total		
Screen size	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**n**
0.4 mm	41.3	9.7	22	14.4	63.3	19.5	3
2 mm	118.3	89.7	85.7	26	204	83.6	3
Probability *	0.63		0.09		0.12		

*On transformed (√) data

Table 5.2.5: Initial and final (1 week) populations of *A. franciscana* retained by 1 mm screen in Experiment 5.8.

Screen Size	Initial Pop./L		1 Week Pop./L		n
	Mean	SE	Mean	SE	
0.4 mm	29.8	2.46	25.9[a]	0.98	3
1.6 mm	25.9	0.98	4.53[b]	1.83	3
Probability	0.22		0.01		

Means sharing a common superscript are not significantly different (p>0.05).

Table 5.2.6: Initial and final (one week) populations of fecund females retained by 1 mm screen in Experiment 5.8.

Screen size	Initial Pop./L		1 Week Pop./L		n
	Mean	SE	Mean	SE	
0.4 mm	3.33[a]	0.58	3.33[a]	0.85	3
1.6 mm	3.87[a]	0.7	1.87[a]	0.77	3
Probability	0.6		0.27		

Means sharing a common superscript are not significantly different (p>0.05).

5.2.3.4 Experiment 5.9: The Effect of Screening (0.4 mm and 1.6 mm) With And Without Brine Recycling, on A.franciscana *Population Growth and Reproductive Output in 1,000 L Outdoor Tanks.*

The Means Table of total population of *A.franciscana*/L retained by 1 mm screen is presented in Table 5.2.7. There was a significant initial difference in overall populations with Treatment 2 tanks having significantly lower numbers but the experiment commenced regardless because the initial populations of adult females were not significantly different (Table 5.2.8). Overall populations, and in particular the population of Treatment 2, declined over the following three days Table 5.2.7). Fecund female population(about 50% of the total adult population) although not significantly different to start with exhibited significant differences between treatments after three days with the population in Treatment 2 significantly lower than the other three treatments. The experiment was abandoned after three days when it

was obvious that the recycling (Treatment 2) was drawing adults and offspring through the screens and populations were collapsing.

Table 5.2.7: Means table of *A.franciscana* population retained by 1 mm screen in Experiment 5.9.

Treatment	Initial No./L			Final (3 day) Number/L		
	Mean	SE	n	Mean	SE	n
1. Control	35.2[a]	1.16	3	25.1[a]	1.16	3
2. Recycle	26.1[b]	1.16	3	6.5[b]	0.81	3
3. 0.4mm O'Flow	33.4[a]	3.2	3	21.9[a]	5.1	3
4. 1.6mm O'Flow	32.9[a]	1.97	3	25.7[a]	0.7	3
Probability	0.029			0.0004		

Means sharing a common superscript are not significantly different (p>0.05).

Table 5.2.8: Means Table of fecund females retained by 1 mm screen in Experiment 5.9.

Treatment	Initial No./L			Final (3 day) Number/L		
	Mean	SE	n	Mean	SE	n
Control	10.87	2.31	3	11.47[a]	1.07	3
Recycle	8.4	0.81	3	1.87[b]	0.59	3
0.4mm O'Flow	12.7	2.9	2	8.03[a]	3.53	2
1.6mm O'Flow	14.9	3.3	3	10.8[a]	3.8	3
Probability	0.2			0.003		

Means sharing a common superscript are not significantly different (p>0.05).

5.2.3.5 Experiment 5.10: The Reproductive Response of A. franciscana *to Selective Removal of Offspring Via Overflow Screens.*

This experiment consisted of two treatments in quadruplicate. Many animals were, however, lost through a torn screen in one of the 150 μm tanks. These data have been excluded from the results.

The population change of *A.franciscana* over the five week trial is shown in Figure 5.2.2. The initial decline in population in both treatments was caused by the death of many newly captured animals. After this time the populations in tanks with 150 μm screens increased and there was a steady decline in the tanks with 1 mm screens where recruitment was prevented.

The total reproductive output per female is shown in Figure 5.2.3 and tends to follow an inverse trend to the population trends with the exception of the final week where reproductive output per female in tanks with 150 μm screens increased. This coincided with an increase in microalgae food in the exchange water from about 500,000 *D.salina* cells/mL to 2,000,000 *D.salina* cells/mL.

There was no significant difference in female *A.franciscana* populations, total reproductive output or cyst production between treatments at the start of the trial (Figures 5.2.8, 5.2.3, 5.2.4). The population differences between treatments were highly significant at the end of the trial (Figure 5.2.2) but reproductive output (Figure 5.2.3) and final cyst production (Figure 5.2.4) were not significantly different. This contrasts with the situation in week four where, although there were significantly less females in the tanks with 1mm screens (Figure 5.2.8), overall reproductive output/L was significantly higher (Figure 5.2.9) due to significantly increased reproductive output per female (Figure 5.2.2). Cyst production/female was significantly higher in the 1 mm screen treatment after four weeks (Figure 5.2.6).

The mean physico-chemical parameters were not obviously different between treatments at either the start or end of the trial (Table 5.2.10) although mean pH was slightly higher in the tanks with large screen size.

The final wet weights of animals are shown in Table 5.2.11 and show that biomass is much greater in tanks equipped with small screens.

Table 5.2.9 Mean Initial and final Physico-chemical parameters in Experiment 5.10.

Physico-chemical Parameters	**Initial**		**5 weeks**	
	150 μm	1,000 μm	150 μm	1,000 μm
pH	8.77	8.76	8.04	8.15
Density (g/mL)	1.1202	1.1200	1.1368	1.1370
Temp. Range	17 - 26°C			
O_2 ppm (1500 hrs)			2.65	2.65

Highly significant difference between treatments ($p<0.001$).

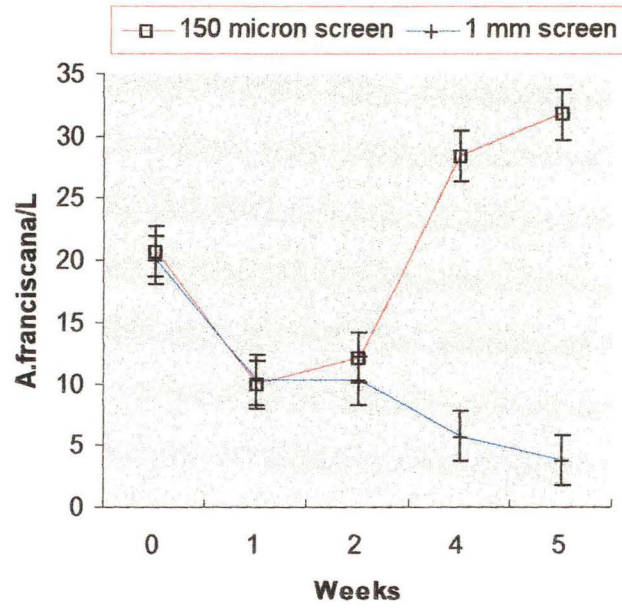


Figure 5.2. 2: Population of *A.franciscana*/L (Mean ± SE, n=4) in Experiment 5.10. SE based on mean square error.

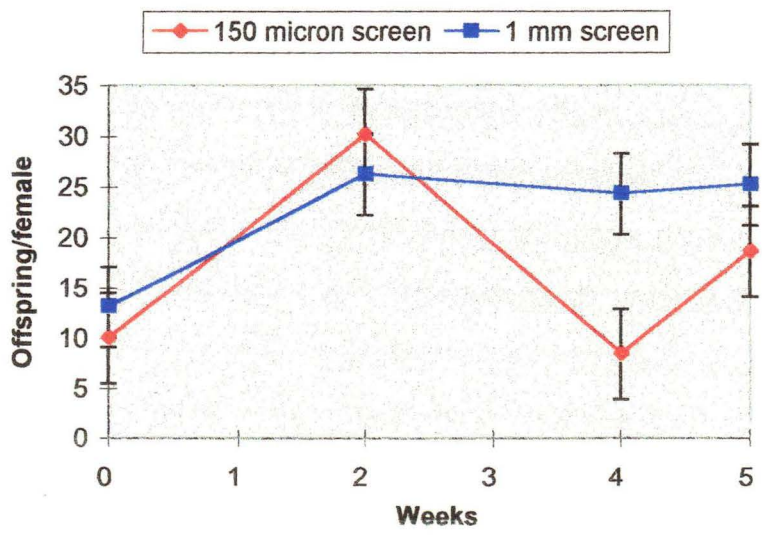


Figure 5.2. 3: Total reproductive output/female (Mean ± SE, n=4) over time in Experiment 5.10. SE based on mean square error

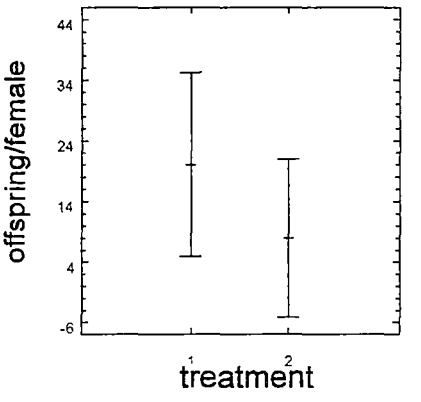


Figure 5.2.4: Mean ± 95% LSD intervals for initial cyst production/female in Experiment 5.10. P=0.32

Treatment Codes for Figures 5.2.4 - 5.2.10

1= 150μm screen

2=1mm screen

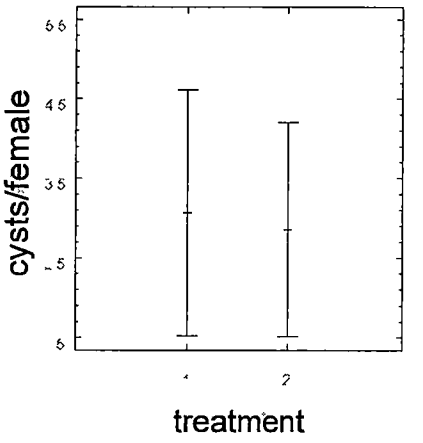


Figure 5.2.5. Mean ±95% LSD intervals for final cyst production/female in Experiment 5.10. P=0.86.

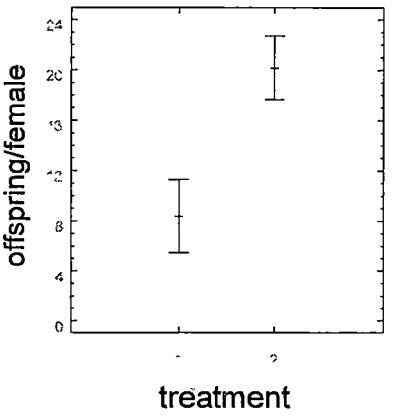


Figure 5.2.6. Mean ±95% LSD intervals for reproductive output/female after four weeks in Experiment 5.10. P=0.003.

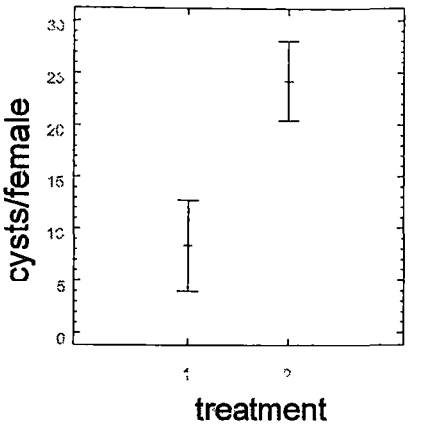


Figure 5.2.7. Mean ±95% LSD intervals for cyst production/female after four weeks in Experiment 5.10. P=0.004

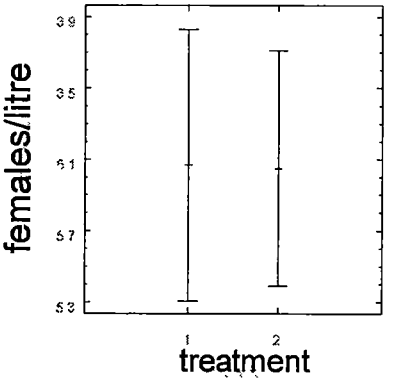


Figure 5.2.8. Mean ±95% LSD intervals for initial population of females/L in Experiment 5.10. p=0.98.

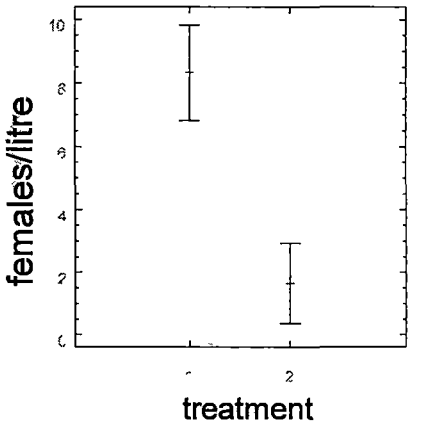


Figure 5.2.9. Mean ±95% LSD intervals for females/L in Week 4 in Experiment 5.10. $p < 0.001$.

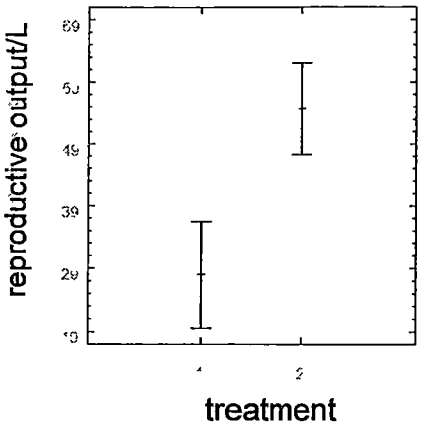


Figure 5.2.10. Mean±95% LSD intervals for reproductive output/L in Week 4 in Experiment 5.10. p= 0.002.

5.2.4 Discussion

The screening experiments discussed in this section (5.2) were undertaken from first principle experimentation as there is a scarcity of similar experiments reported in the literature.

In Experiment 5.6 the fine mesh used as a secondary screen clogged quickly with detritus and prevented throughflow of brine. This may have contributed to the lack of effectiveness in limiting population expansion in any treatment and of the close similarities in final populations of animals at the end of the trial (Table 5.2.1). It was apparent that a different screening method needed to be found.

In Experiment 5.7 the 2 Way ANOVA table of *A.franciscana* retained by a 1 mm screen (Table 5.2.2) showed there was a significant decline in population due to screen size and a significant overall reduction in population over the one week trial and a significant interaction between these factors. Fecund female (those with ovisacs) population on the other hand did not change significantly (Table 5.2.3) although there was some (not significant $p>0.05$) population reduction indicating that although most females were too large to pass through the 2 mm screens some were lost. Those remaining were all large (>1 cm long). The picture is clarified by Figure 5.2.1 which presents the mean and standard error data for all combinations of screen size and time for both total population (retained by a 1 mm screen) and adult females (with ovisacs) graphically.

The results for reproductive output (Table 5.2.4) were variable and more females were needed to reduce this variability. It does appear, however, that there was some increase in reproductive ouput per female in tanks with fewer animals.

In Experiment 5.8 the total population of animals in tanks equipped with 1.6 mm screens had reduced greatly after one week (Table 5.2.5) but the fecund female population was not significantly different (Table 5.2.6). Mean numbers of fecund females were, however, lower and some smaller females may have been lost. Females reach maturity at a length of about 8 mm but continue to grow until a maximum size of about 1.2 cm (Field observations on Dampier strain *A.franciscana*). It appears that a screen size of 1.6 mm, or even 2 mm is small enough to retain mature adults while allowing offspring to pass through. Care must, however, be taken to ensure that the majority of the mature animals are close to full size to avoid the possibility of losing significant numbers of broodstock.

In Experiment 5.9 the population of fecund females was about the half the size of the total population in most tanks (Table 5.2.7 and 5.2.8) indicating that most of the *A.franciscana* retained by the 1 mm screen were adults. The populations of both total animals and fecund females did not change substantially in either the control tanks or the tanks equipped with a 1.6 mm overflow screen. The reduction of population in tanks fitted with 400 μm overflow screens was due to loss of animals when the tanks overflowed, particularly in one tank. This was due to the screens being too small to cope with the brine flow, despite air curtain cleaning.

A great reduction in animal populations occurred in all tanks where the brine was recycled through the 1.6 mm screens. This was probably due to an increased flow rate, compared to the overflow screens, resulting in animals being drawn through the screens.

The experiment confirmed that a screen size of 1.6 mm was sufficient to retain adult *A.franciscana* under conditions of gravity flow. Active pumping, however, seems to draw the adults through this sized screen and other this situation a smaller screen size may be required to retain adults. The loss of food through the overflow system can be minimised by overflowing brine after most food has been consumed, or by recycling the overflowed brine back through the tanks.

Experiment 5.10 confirmed that selective screen sizes can limit the population growth of *A.franciscana* (Figure 5.2.2, Table 5.2.10). This in turn limits competition between animals and ensures that reproducing females receive more food than they would in a more crowded environment. This should result in greater fecundity for these individuals and up until Week 4 this was the clear pattern (Figures 5.2.3, 5.2.6, 5.2.7).

The increase in reproductive output among the highly competitive animals in tanks with 150 μm screens in the final week can be explained by food supply increases. Each tank was receiving 500 L of *D.salina* culture brine each day. During the course of the experiment the microalgae culture pond became contaminated with *A.franciscana* and the concentration of *D.salina* cells declined to about 500,000 cells/mL in Week 4 from the original 2,000,000 cell/mL concentration. (All incoming culture brine was

screened through a 150 µm screen to prevent any contaminating *A.franciscana* possibly in the microalgae pond entering the experimental tanks). In the fifth and final week of the experiment *D.salina* culture at the original 2,000,000 cells/mL concentration was accessed from a different microalgae pond that had been established during the decline of the first pond. This increase in food input stimulated reproductive output among highly competitive animals in tanks with small screens and this increased fecundity had not yet manifested itself in greatly increased population size. Increased food input did not, however, appear to stimulate reproductive output among *A.franciscana* already receiving high levels of food in tanks with 1 mm screens (Figure 5.2.3). These animals appear to have reached a reproductive peak of about 25 offspring/female/3 days in the second week (Figure 5.2.3). Three day intervals were chosen for experimental purposes on the basis of results obtained from Experiment 5.1 and other earlier experiments. Old World bisexual *Artemia* are characterised by small broods (20 to 30 offspring/brood) and relatively short brood intervals (3.5 to 4.8 days) (Lenz, 1987). Most of the offspring were encysted (Figures 5.2.6 and 5.2.7) and *Artemia* with a high proportion of their offspring encysted generally have low reproductive output and short life-spans (Lenz and Browne, 1991) Reproductive output did not increase beyond this level despite a decline in the population of these tanks over the next few weeks and may be a maximum. This figure is close to the reproductive maxima/female achieved in previous experiments (Experiment 5.4) and in a 0.1 ha experimental pond (Chapter 6).

The reproductive output/female must be considered in conjunction with the population of reproducing females. This population, although initially not significantly different (Figure 5.2.8), was greatly different after four weeks (Figure 5.2.9). The much greater reproductive output per female at this time did, however, result in much greater reproductive output/L despite the smaller population in tanks with large screens (Figure 5.2.10). This assumes that the reproductive output of females removed from the tanks is representative of the females remaining within the tank.

The physico-chemical parameters at the beginning of the experiment and after five weeks are shown in Table 5.2.9. There are no obvious differences between treatments so it is probable that observed changes in population level and reproductive output are the result of the screen sizes.

There was a highly significant difference in final wet weight of *A.franciscana*/treatment at after five weeks (Table 5.2.10). This was expected due to the large difference in *A.franciscana* populations between treatments (Figure 5.2.2).

5.2.5 Conclusion

Selective screening of *A.franciscana* culture can be effective in both limiting population expansion and stimulating reproductive output. Reproductive output for the Dampier strain *Artemia* did, however, appear to peak at a maximum of about 25 offspring/female/3 day brood interval. In a practical situation an optimum balance would be sought between *A.franciscana* population size and reproductive output in order to maximise reproductive output/volume of culture brine.

The six month longevity of *Artemia* (Sorgeloos et al. 1986) and the continuous recruitment of young individuals despite screening makes it extremely unlikely that the population will decline unsustainably due to excessively efficient removal of offspring and in practice in a 0.01 ha experimental pond operated over many months (Chapter 6) this did not happen. If, however, it was found necessary to reduce the efficiency of screening this could be achieved by reducing the flow rates through the screens until an optimum balance was achieved.

5.3 The Reproductive Response Of A.fanciscana *To Salinity*

5.3.1 Introduction

It is widely reported that Artemia spp. are ovoviviparous in low salinity ponds but oviparous in high salinity ponds (Davis, 1980; Sorgeloos et al., 1986; Tackaert and Sorgeloos, 1991; Brown et al., 1992). The reports largely originate from observations that brine ponds containing Artemia spp. and high salinity brine tend to have obvious accumulations of cysts on the periphery. These reports contradict the findings of Berthelemy-Okazaki and Hedgecock (1987) who found that oviparity significantly decreased with increasing salinities in two parallel trials encompassing salinities from 15 to 80 ppt in one trial and 30 to 180 ppt in another. Accumulations of cysts do predominantly occur in ponds of higher brine density but many of these cysts originate from upstream ponds. The low to middle salinity (brine density 1.07-1.14 g/mL) ponds at Dampier Salt always contain suspended Artemia cysts (routine biological monitoring data from the saltfield. They are initially suspended in low density brine and become increasingly buoyant as the brine density increases resulting in their accumulation on the periphery of more saline ponds. Fecund female Artemia freshly captured from the Dampier saltfield, and allowed to spawn in beakers in the laboratory, generally produce about 25% of their offspring as cysts throughout the brine density range 1.07 g/mL to 1.16 g/mL (personal observation). The course of reproduction in these animals was set prior to capture.

Details of four experiments testing the reproductive response of A.franciscana to various brine densities, density-shock and various quality brines are included below.

5.3.2 Methods

5.3.2.1 Experiment 5.11: The Reproductive Response of A.franciscana *to Brine Density.*

A cohort of A.franciscana was reared to maturity in a 500 mL beaker in the laboratory on a diet of D.viridis. A pair of mature animals was placed in each of twelve labelled 60 mL beakers containing 50 mL of four density brines in triplicate. The densities were 1.024 g/mL, 1.08 g/mL, 1.10 g/mL and 1.16 g/mL. Eight million D.viridis cells were added each day to each of twelve parallel labelled beakers containing fresh brine. The animals were transferred daily to the new beakers with a pipettor and the old brines filtered through gridded GF/C filter papers per day to capture offspring which were counted under a dissection microscope. These offspring were not returned to the beakers. The experiment ran for 18 days.

5.3.2.2 Experiment 5.12: The Reproductive Response of A.franciscana *to Brine Density and Brine Density-Shock.*

Experiment 5.11 demonstrated a pattern of reduced oviparity with increasing density and maximum reproductive output at a brine density of 1.08 g/mL. A repeat experiment was designed to confirm the initial findings in the extended brine density range 1.08 g/mL to 1.16 g/mL and to determine the effect of salinity-shock on reproductive output. This last factor was considered relevant due to widespread reports of obvious cysts in ponds following rain at a number of saltfields and similar personal observations at the Dampier Salt Limited saltfield at Dampier. Salinity shocks have been reported as effective in switching *Artemia* populations towards cyst production (Tackaert and Sorgeloos, 1991).

The experiment encompassed four treatments in quadruplicate. Each of 16 labelled 100 mL plastic beakers were filled with GF/C filtered brine collected directly from the saltfield. One pair of reproductively mature animals, collected from saltfield brine of density 1.10 g/mL (where highest *Artemia* populations naturally occur) was placed in each beaker. The brine density of treatments was as follows:

1. 1.0 8 g/mL.
2. 1.10 g/mL.
3. 1.16 g/mL.
4. Daily alternation from 1.08 to 1.16 g/mL.

Two sets of beakers were used (as in Experiment 5.11) with animals being transferred daily to fresh brine to which 2 mg wheat pollard and 2 million *D.viridis* cells had previously been added. Old brines were filtered through GF/C filter papers and a count made under a dissecting microscope of all produced cysts and nauplii. These were not returned to the beakers. The experiment ran for fifteen days.

5.3.2.3 Experiment 5.13: The Reproductive Response of A.franciscana *to Salinity-Shock.*

Experiment 3 was conducted after Experiment 5.12 showed no significant effect on mode of reproduction or reproductive output following salinity-shock. Experiment 3 utilised animals pre-adapted to both high and low salinity brine.

Brine of density 1.03 g/mL from a newly flooded pond while still free of fish but containing numerous *A.franciscana* was collected along with a sample of *A.franciscana* from the pond. Brine and *A.franciscana* were also collected from an adjacent pond with brine density 1.12 g/mL.

Eight 1 L plastic jars were filled with each type of filtered brine and five pairs of *A.franciscana* originating from the same brine were placed in each jar with extreme care being taken to avoid contamination with any cysts or nauplii. 10 mg of finely ground wheat pollard and 5 million *D.salina* cells were added to each beaker each day on the basis of previous experiments on optimum feed rates (Chapter 3.2). The animals were fed for four days and allowed to acclimatise before being transferred to identical beakers containing fresh brine. They were then fed for a further four days before being transferred again. The second transfer involved placing one set of low salinity animals into high salinity brine and one set of high salinity animals into low salinity brine. The experiment ran for a further four days. A count of all produced cysts and nauplii was made at each transfer.

The treatments in quadruplicate were as follows (Low Salinity (LS) is density 1.03 g/mL and High Salinity (HS) is density 1.12 g/mL:

First 4 days	**Second 4 days**
1. LS Brine + LS animals	LS brine + LS animals
2. LS brine + LS animals	HS brine + LS animals
3. HS brine + HS animals	HS brine + HS animals
4. HS brine + HS animals	LS brine + HS animals

5.3.2.4 Experiment 5.14: The Effect of Ionic Composition on A.franciscana *Reproduction.*

An experiment was designed to determine whether brines of different ionic composition but of the same density (g/mL) had an influence on the reproductive response of *A.franciscana*. This experiment was undertaken to determine the flexibility in preparing culture brine for *A.franciscana* culture. Three brines, all of density 1.10 g/mL were prepared as follows:

1. Seawater plus sodium chloride.
2. Field brine (seawater concentrate).
3. Seawater plus high density (1.3 g/mL) bitterns brine.

The calcium content of these mixtures using the data of Baseggio (1972) (Appendix 1) was 0.35, 1.3 and 0.4 g/L respectively. Two pair of mature *A.franciscana* were added to quadruplicate 50 mL samples of each of the above brines in 60 mL plastic beakers. All animals were transferred to clean beakers of fresh brine after one week and again after two weeks. Old brines were filtered through gridded pre-filter papers and a count made of all produced offspring by methods described earlier.These were not returned to the beakers.

5.3.3 Results

The results of Experiment 5.11 are shown in Table 5.3.1. All animals in the 1.16 g/mL density brine treatment died and there was one death in the 1.024 g/mL density brine treatment. These data have been excluded. Greatest reproductive output occurred in the 1.08 g/mL density brine treatment and there was a clear and highly significant trend of decreasing oviparity with increasing salinity.

Table 5.3.1: Total reproductive output and percent cystation for *A.franciscana* in 50 mL beakers over 18 days on a diet of *D.viridis* in Experiment 5.11.

Densityg/mL	Offspring/Female/18 days.			Overall % cysts		
	Mean	S.E.	n	Mean	S.E.	n
1.024	29.5^{a}	6.5	2	97.2^{a}	2.8	2
1.08	142^{b}	11.5	3	32.7^{b}	4.84	3
1.10	88.3^{c}	12.2	3	18.7^{c}	3	3
Probability	0.004			<0.001		

Means sharing a common superscript are not significantly different (p>0.05).

In Experiment 5.12 animal deaths occurred in one replicate of each of three treatments during the experiment and these data have been excluded. There were no significant differences ($p>0.05$) between treatments with either total reproductive output or percent oviparity (Table 5.3.2). Variation among replicates was very high.

Table 5.3.2: Total reproductive output and percent cystation of *A.franciscana* in 100 mL beakers over 15 days in Experiment 5.12.

	Offspring/Female/15 d			**Overall % cysts**		
Density g/mL	**Mean**	**S.E.**	**n**	**Mean**	**S.E.**	**n**
1.08	142.7	21.3	3	34.2	18.9	3
1.10	141.7	29.7	3	49.33	23.3	3
1.16	88	15.9	3	25.5	9.2	3
Alternation*	93.8	22.7	4	16.6	8.9	4
Probability	0.26			0.5		

*daily alternation from 1.08 to 1.16 g/mL.

The reproductive output from *A.franciscana* in the first and second 4-day periods (encompassing a full reproductive cycle) of Experiment 5.13 is shown in Table 5.3.3a. In the first 4-day period the reproductive output in Treatment 4 was significantly less than that in Treatments 1 and 2. Treatment 3 reproductive output was also lower in this period (Note = Animals in Treatments 3 and 4 originated from clear high salinity brine with much lower population density of planktonic microalgae than the low salinity brine from which the animals in Treatments 1 and 2 originated). There was no significant difference in total reproductive output between treatments in the second 4-day period. There was no significant change in total reproductive output between the first and second 4 day periods (Table 5.3.3.b).

Treatment four had significantly higher oviparity than other treatments in the first 4-day period (Table 5.3.4). This trend reversed in the second 4-day period with Treatments 1 and 2 having significantly higher oviparity than Treatments 3 and 4 which had high salinity animals.

Table 5.3.3a: Reproductive output/treatment of *A.franciscana* in 1 L plastic jars first and second 4-day period of Experiment 5.13.

Treatment	Day 1 - 4			Day 5 - 8		
	Mean	SE	n	Mean	SE	n
1. LS*a control	148.3^{a}	21.6	4	135.3	12.7	4
2. LSa to HSb	154.5^{a}	22.99	4	128.8	14	4
3 .HSa control	103^{ab}	13.4	4	84.5	21.1	4
4 .HSa to LSb	79.3^{b}	12.3	4	117	12	4
Probability	0.036			0.15		

Means sharing a common supersript are not significantly different (p>0.05).

*LS (Low Salinity)=1.03 g/mL density
HS(High Salinity)=1.12 g/mL density

Table 5.3.3b: Change in Reproductive output/treatment of *A.franciscana* in 1 L plastic jars between first and second 4-day period of Experiment 5.13.

Treatment	Reproductive output change/treatment		
	Mean	SE	n
1. LS*a control	-13	9.7	4
2. LSa to Hsb	-25.7	30.6	4
3. HSa control	-19	27.9	4
4. HSa to LSb	37.8	7.1	4
Probability	0.2		

*LS (Low Salinity)=1.03 g/mL density

HS(High Salinity)=1.12 g/mL density

Table 5.3.4: Percent oviparity/treatment in first and second 4-day period of Experiment 5.14.

Treatment	Day 1 - 4			Day 5 - 8		
	Mean	SE	n	Mean	SE	n
1. LSa control	35.5^{ab}	1.84	4	61.5^{a}	3.9	4
2. LSa to HSb	20.61^{b}	6.86	4	76.3^{a}	9.02	4
3. HSa control	13.7^{b}	4.78	4	16.5^{b}	5.4	4
4. HSa to LSb	55.35^{a}	15.1	4	36.75^{b}	10.48	4
Probability	0.025			0.0007		

Means sharing a common supersript are not significantly different ($p>0.05$).

*LS (Low Salinity)=1.03 g/mL density
HS(High Salinity)=1.12 g/mL density

The total reproductive output in Treatment 2 of Experiment 5.14 was significantly higher than that of Treatment 1 in the first week but in the second week there was no significant difference between treatments (Table 5.3.5). The percentage oviparity between treatments was not significantly different in either the first or second weeks although there was a marked reduction in overall oviparity in all treatments (Table 5.3.6).

Table 5.3.5: Reproductive output/beaker in first and second weeks of Experiment 5.14.

Treatment	First week			Second week		
	Mean	SE	n	Mean	SE	n
1.Bitt.+ SW	14.5^{a}	2.7	4	16.3	2.8	4
2.Salt + SW	28^{b}	1.7	4	21.5	5.1	4
3.Brine Conc.	15.8^{ab}	4.3	4	37.8	14.9	4
Probability	0.023			0.28		

Means sharing a common supersript are not significantly different.($p>0.05$).

Table 5.3.6: Percent Oviparity in first and second week of Experiment 5.14.

Treatment	First week			Second week		
	Mean	SE	n	Mean	SE	n
1.Bitt.+ SW	53.8	18.6	4	7.3	5.98	4
2.Salt + SW	76.3	10.8	4	34.8	22.2	4
3.Brine Conc.	32.2	21.8	4	18.12	11.82	4
Probability	0.27			0.46		

5.3.4 Discussion

In Experiment 5.11 the trend of decreasing oviparity with increasing salinity was consistent with the findings of Berthelemy-Okazaki and Hedgecock, 1987 and of Williams and Mitchell (1992) in their study with parthenogenetic South African *Artemia*. The maximum reproductive response in the 1.08 g/mL density treatment was at the lower end of the 100 to 170 ppt salinity range found to maximise *Artemia* reproductive output in a study by Wear and Haslett (1987) for Lake Grassmere *Artemia* when brine temperature was between 20 and 28^{o}C. It is slightly above the 75 ppt salinity that maximised reproductive response with parthenogenetic South African *Artemia* in the study by Williams and Mitchell (1992).

The results of Experiment 5.12 failed to conclusively confirm the results of Experiment 5.11. There were no significant differences in either reproductive output or oviparity with brine density or with salinity-shock (Table 5.3.2) although salinity shock has been reported as an effective way of switching *Artemia* populations towards cyst production (Tackaert and Sorgeloos, 1991). There were no significant differences between treatments with either total reproductive output or percent oviparity (Table 5.3.2). However, variability among replicates was much higher than in Experiment 5.11 and the trends for reproductive output and proportion cysts were consistent.

In Experiment 5.13 the initial reproductive output of animals from and in low salinity brine was significantly greater than their high salinity counterparts (Table 5.3.3), consistent with Experiment 1 and the findings of Berthelemy-Okazaki and Hedgecock, 1987. This difference was not, however, significant in the second 4-day period (Table 5.3.3) although the trend was similar. The first 4-day period occurred after a four day acclimatisation and as no treatments had been applied at this time it is probable that the difference in reproductive output was due to residual better condition in the low salinity animals. These originated from a pond with higher available food than the high salinity animals and probably had

more energy reserves. There was no significant difference in reproductive output between treatments in the second 4-day period.

There was a significant difference in percent oviparity between treatments in both the first and second 4-day period (Table 5.3.4). In the first 4-day period Treatment 4 had significantly higher oviparity than either Treatment 2 or 3. This demonstrates the innate variability of *A.franciscana* because animals in Treatment 3 had originated from the same source as animals in Treatment 4 and at that stage had been in identical conditions. In the second 4-day period there was a highly significant difference in oviparity between low salinity animals and high salinity animals regardless of salinity-shock. Once again the variability of *A.franciscana* is illustrated by the fact that percent oviparity in Treatment 1 doubled even though the conditions under which these animals were housed had not changed.

In Experiment 5.14 there was a significantly higher reproductive output from Treatment 2 in the first week of the experiment (Table 5.3.5). This pattern did not persist in the second week. It appears that the ionic composition of the brine has little effect on reproductive output or percent oviparity at a brine density of 1.10 g/mL (Table 5.3.5 and 5.3.6). This indicates that *A.franciscana* culture brines can be derived from seawater to which either salt or bitterns has been added as well as from normal seawater concentrate brine. This in turn imparts a great deal of flexibility in establishing culture systems. However, the variability among replicates was not conducive to detecting even large differences among treatments (low power).

It appears on the basis of the above experiments and personal observations on the ponds at Dampier Salt, that salinities within the range 1.08 to 1.16 g/mL do not have a marked impact on Dampier strain *A.franciscana* reproductive output or oviparity. High salinities in particular do not appear to produce oviparity as reported for other strains of *Artemia* (Davis, 1979; Sorgeloos et al. 1986; Browne et al., 1992). It is likely that cysts produced in low salinity ponds remain in suspension until the brine density increase makes them buoyant enough to float leading to their accumulation in high salinity ponds. Analysis of the condition of cysts conspicuous in rafts in the ponds at Dampier Salt Limited after rain has revealed that they are poor quality and consistent with old cysts washed from peripheral rocks where they were previously washed and not fresh cysts produced in response to salinity-shock. Some *A.franciscana* cysts are always suspended in the brine column of the ponds at Dampier Salt, particularly when conditions are windy and particularly in low salinity brines where *A.franciscana* first occur.

5.4 The Reproductive Response of A.franciscana *to Direct Sunlight and Starvation.*

5.4.1 Introduction

A number of the laboratory experiments described in Sections 5.1-5.3 were conducted using the Dampier strain of *A.franciscana* as experimental animals. These animals often demonstrated a marked change in reproductive mode from being primarily oviparous to ovoviviparous during the course of the experiments irrespective of treatment (e.g. Chapter 5.3, Experiment 5.14). The reasons for this change were not clear but were thought to be caused by differences in environmental conditions. In their review paper on *Artemia* ecology, Lenz and Browne (1991) suggest that although there are genetic influences on encystment rates there are also large environmental influences. Ovoviviparity prevails when conditions are highly favourable and a switch towards oviparity occurs when conditions become unfavourable (Lenz and Browne,1991).

Two major differences were the high food level and low light conditions in the laboratory compared to the low food and high light experienced in the clear field brine from which they were caught. Preliminary experimentation involving both the starvation of animals and exposing them to full sunlight conditions did not return reproductive mode to a primarily oviparous form.

Details of two experiments designed to determine the reproductive response of *A.franciscana* to starvation and a combination of starvation and high light intensities are included below. The first experiment was a range-finding exercise designed to determine whether starvation had a marked impact on the percent oviparity of primarily ovoviviparous animals from Experiment 5.12. The second experiment was designed to determine whether high light intensities in combination with starvation affected oviparity.

5.4.2 Methods

5.4.2.1 Experiment 5.15. The reproductive response of A.franciscana *to starvation.*

Well fed animals from Experiment 5.12 (Section 5.3) were retained at the completion of that experiment. They were subjected to increasingly longer periods of starvation and their reproductive response noted in the following experiment. The basic methods were identical to those described for Experiment

5.12. Starvation periods began with a three day break without food to ensure all females would be ovulating at least once in this period and then the animals were fed the equivalent of three days food. They were then left without food for another four days with the intention of increasing the starvation period by one day between consecutive feedings. Most of the females had, however, died at the end of the second starvation period.

5.4.2.2 Experiment 5.16: The Reproductive Response of A.franciscana *to Food Level and Light Intensities.*

Five pairs of reproductively mature *A.franciscana* captured from the field were added to each of 16 by 1 L plastic beakers containing 500 mL of filtered brine of density 1.10 g/mL. Four treatments were then applied in quadruplicate with beakers randomised.

The treatments were as follows:

1. Animals housed in laboratory and fed high rate of $1.0x10^6$ *D.salina* cells + 1 mg wheat pollard/animal/day.
2. Animals housed in laboratory and fed low rate of $0.5x10^6$ *D.salina* cells + 0.5 mg wheat pollard/animal/day.
3. Animals housed outdoors and fed high rate of $1.0x10^6$ *D.salina* cells + 1 mg wheat pollard/animal/day.
4. Animals housed outdoors and fed low rate of $0.5x10^6$ *D.salina* cells + 0.5 mg wheat pollard/animal/day.

Food was added daily by techniques described in previous experiments. All animals were transferred to beakers containing fresh filtered brine every three days and a total count made of all produced cysts and nauplii after filtering the brine through GF/C filter paper. These were not returned to the beakers. The experiment ran for two weeks.

5.4.3 Results

5.4.3.1 Experiment 5.15. The Reproductive Response of A.Franciscana *to Starvation.*

Experiment 5.15 (Table 5.4.1) shows that there were no significant differences between salinity treatments in either total reproductive output or percent oviparity after one weeks starvation. Unfortunately the death of animals caused a premature end to the experiment so determination of change in percent oviparity over time was not possible. It was apparent, however, that mode of reproduction was essentially ovoviviparous after one week of periodic starvation.

Table 5.4.1: Offspring produced during one week of periodic starvation in Experiment 5.15

Treatment	Reprod. Output/Beaker			% Oviparity		
	Mean	SE	n	Mean	SE	n
1.05 g/mL	102.8	26.3	4	6.7	4.7	4
1.08 g/mL	97.8	32.7	4	6.2	2.9	4
1.16 g/mL	69.8	6.8	4	36.5	17.8	4
Alt.1.16-1.05 g/mL	78.3	13.6	4	7.7	7.7	4
Probability	0.69			0.15		

5.4.3.2 Experiment 5.16: The Reproductive Response of A.Franciscana *to Food Level and Light Intensities.*

There were no significant differences in total reproductive output data in Experiment 5.16 (Table 5.4.2.) for either light intensities or food level although food level (p=0.052) was close to being significant with animals fed at higher levels producing more offspring. There was no significant interaction.

The mode of reproduction was not significantly affected by either food level or light intensities (Table 5.4.3) with mean oviparity of about 40% in all treatments. Light intensities in the laboratory were under 1,000 lux at all times and up to 25,000 lux outdoors at noon. There was no significant interaction.

Table 5.4.2: Means table and probabilities for two way ANOVA for total offspring /beaker during Experiment 5.16.

	Prob.	Factor	Mean	SE (pooled)	n
Light	0.51	Indoor	407.8	42.8	8
		Outdoor	449.4	42.8	8
Food	0.052	High food	493.4	42.8	8
		Low food	363.8	42.8	8

Table 5.4.3: Means table and 2 way ANOVA probabilities for percent oviparity in Experiment 5.16.

	Probability	Factor	Percent Oviparity		
			Mean	SE (pooled)	n
Light	0.43	Indoor	39.2	6.4	8
		Outdoor	46.8	6.4	8
Food	0.87	High food	43.7	6.4	8
		Low food	42.2	6.4	8

5.4.4 Discussion

Some batches of soft cysts (i.e. with partially formed shells)and very occasional normal cysts were produced in all treatments prior to maternal deaths which all occurred on the final day of the second starvation period when Experiment 5.15 was terminated. There was no change back to oviparity with the main reproductive output continuing to be nauplii, with the possible exception of the 1.16 g/mL brine density treatment where substantial numbers of cysts were produced. There was no indication of a switch to oviparity when conditions became unfavourable as suggested by Lenz and Browne (1991) in their review. It is possible that conditions became unfavourable too quickly.

The fact that the male partners survived while the females died could indicate that the very last reserves of the females went into reproductive output before they starved.

Outdoor culture with high light intensities from direct sunlight did not significantly affect either mode of reproduction or total reproductive output (Tables 5.4.2 and 5.4.3). There was also no significant effect on mode of reproduction or reproductive output with food level although additional food may have stimulated total reproductive output (Tables 5.4.2 and 5.4.3). It is likely that the animals in the low feeding level treatment were not severely food limited even though they were only receiving half the food volume of animals at the high feeding level.

5.5 The Reproductive Response of A.franciscana *to Oxygen Stress in the Presence of Iron*

5.5.1 Introduction

With the exception of Experiment 5.11 in Chapter 5.3 which demonstrated that low salinity favours cyst production (Table 5.3.1) the previous experiments in this chapter (5.1 - 5.4) failed to conclusively identify factors controlling the switch in reproductive mode from live young to cysts. A combination of oxygen stress in the presence of iron has been reported as being highly effective in stimulating oviparity in some studies (Versichele and Sorgeloos, 1980 and; Balasundaran and Kumaraguru, 1987) while other studies were not conclusive (Berthelemy-Okazaki and Hedgecock, 1987). This needed to be investigated for Dampier strain *A.franciscana*.

The following experiment was based broadly on the work of Versichele and Sorgeloos (1980) but was conducted on a large scale, outdoors, and using nitrogen flushing to reduce oxygen levels rather than discontinuous aeration. Discontinuous aeration techniques were not effective in rapidly reducing oxygen levels in 1,000 L tanks in preliminary trials.

5.5.2 Methods

Twelve 1,000 L tanks were filled with brine of density 1.10 g/mL and fertilised with 0.01 g/L Aquasol. The brine was vigorously aerated using air delivered through perforated PVC pipe fed from an axial air blower and inoculated with *D.salina*. The brine became green within two weeks and contained about 200,000 *D.salina* cells per mL. *A.franciscana* nauplii were hatched from cysts collected from the saltfield and placed in a 200 L plastic tub containing aerated seawater. After 48 hours the population density of nauplii was determined by drawing the seawater into a 1 mL pipette and counting the nauplii against a light. About 50,000 nauplii were then added to each tank by dispensing an appropriate aliquot to give an initial population of about 50 animals/L. 20 g of finely ground wheat pollard was added to each tank each day and the animals were allowed to mature over two weeks. The population of both total animals and mature females retained by a 1 mm screen was then determined for each tank. This was done by vigorously agitating the brine with an oar before taking a 1 L sample which was then poured through a 1 mm mesh. Retained animals were counted and returned to the tank. The process was repeated five times per tank. Populations of both mature females and total animals were also

determined at the end of the experiment (one week). The following treatments were applied to the tanks at random in triplicate:

1. Well aerated without iron.
2. Well aerated with iron.
3. Oxygen stressed without iron.
4. Oxygen stressed with iron.

Iron was added as FeEDTA to add 5 ppm Fe to the brines requiring iron at the beginning of the seven day trial and there was no water exchange. Iron levels were determined by titration at the end of the trial. Oxygen stress was applied hourly by using nitrogen gas to flush oxygen from the brine column. The method was as follows. All tanks received air delivered through perforated PVC pipes fed from an axial blower for 55 minutes. A timer then automatically switched off the air blower and at the same time activated both a small compressor pump and a solenoid valve in an air line leading to a nitrogen cylinder. This enabled nitrogen gas to enter the eight oxygen stressed tanks as a fine mist of bubbles through "air/water wik" tubing. At the same time the eight tanks containing well oxygenated animals received air from the air compressor also delivered through "air/water wik" tubing. The nitrogen flushing proceeded for 5 minutes before the timer reactivated the air blower and switched off the compressor pump and solenoid valve. Oxygen levels in the nitrogen flushed tanks declined from about 6.5 ppm (meter reading on calibrated YSI model 57 oxygen meter, Appendix 2) to about 2.5 ppm and returned to 6 ppm within 5 minutes. The oxygenated tanks remained at a meter reading of about 6.5 ppm. The reproductive output for each treatment was determined at the beginning and end of the trial (one week) by capturing 20 fecund females per tank and allowing them to spawn in filtered brine in the laboratory. This brine was then filtered onto gridded filter papers and a total count made of nauplii and cysts.

5.5.3 Results

There was no significant difference in the initial population of total animals but final population numbers were too variable to be meaningful (Table 5.5.1). The populations of fecund females at the beginning and end of the trial (Table 5.5.2) were not significantly different between treatments. The total reproductive output per 20 females was initially not significantly different between treatments, but after one week it was significantly different with higher reproductive output in tanks with iron (Table 5.5.3). There was a similar pattern for cyst production (Table 5.5.4). The final percent oviparity was not significantly different between treatments with primarily oviparous reproduction in all cases (Table 5.5.7.). The data

for initial per cent oviparity is shown but these are unacceptably variable due to a single outlier data point of 17% with all other figures over 80% preventing conclusive analysis. Neither brine density (Table 5.5.5) or brine pH (Table 5.5.6) were significantly different ($p>0.05$) between treatments at the beginning or end of the trial. Iron was undetectable in all tanks at the end of the trial, including those that had initially received 5 ppm chelated iron.

Table 5.5.1: Initial and final populations of *A.franciscana*/L.

	Initial Population/L			Final Population/L			
Level	**P**	**Mean**	**SE (Pooled)**	**P**	**Mean**	**SE (Pooled)**	**n**
A: Oxygen	0.61			0.42			
stressed(-)		22.5	1.22		25.6	2.58	6
unstressed(+)		23.4	1.22		22.45	2.58	6
B: Iron	0.85			0.13			
-		22.8	1.22		27.1	2.58	6
+		23.13	1.22		20.97	2.58	6
AB (O_2/Iron)	0.22			0.86			
1. - -		21.2	1.73		28.33	3.65	3
2. - +		23.8	1.73		22.87	3.65	3
3. + -		24.4	1.73		25.87	3.65	3
4. + +		22.5	1.73		19.07	3.65	3

Codes for tables 5.5.1 - 5.5.7

O_2/Iron

1.-- (oxygen stressed, no iron)
2.-+(oxygen stressed with initial iron)
3.+-(always air and no iron)
4.++(always air and initially iron)

Table 5.5.2: Initial and final adult female population/L

Level	Initial Females			Final Females			
	P	Mean	SE (pooled)	P	Mean	SE (pooled)	n
A: Oxygen	0.94			0.75			
stressed(-)		8.03	0.9		7.53	1.67	6
unstressed(+)		7.93	0.9		6.73	1.67	6
B: Iron	0.48			0.81			
-		8.47	0.9		6.83	1.67	6
+		7.5	0.9		7.43	1.67	6
AB (O_2/Iron)	0.22			0.77			
1. - -		7.67	1.28		6.87	2.37	3
2. - +		8.4	1.28		8.2	2.37	3
3. + -		9.27	1.28		6.8	2.37	3
4. + +		6.6	1.28		6.67	2.37	3

Table 5.5.3: Initial and final reproductive output/20 females.

Level	Initial Offspring/20 Females			Final Offspring/20 Females			
	P	Mean	SE (pooled)	P	Mean	SE (Pooled)	n
A: Oxygen	0.58			0.37			
stressed(-)		158	48.8		65.83	18.86	6
unstressed(+)		199	48.8		91.83	18.86	6
B: Iron	0.72			0.04			
-		191	48.8		46.67	18.86	6
+		165	48.8		111	18.86	6
AB (O_2/Iron)	0.11			0.59			
1. - -		108.7	69		41.3	26.7	3
2. - +		207.3	69		90.3	26.7	3
3. + -		274.7	69		52	26.7	3
4. + +		123.3	69		131.7	26.7	3

Table 5.5.4: Initial and final cyst production/ 20 females

Level	Initial Cysts/20 Females			Final Cysts/20 Females			
	P	Mean	SE (pooled)	P	Mean	SE (pooled)	n
A: Oxygen	0.64			0.65			
stressed(-)		146	26.8		64	16.5	6
unstressed(+)		127.2	26.8		75	16.5	6
B: Iron	0.37			0.03			
-		118.2	26.8		39.3	16.5	6
+		155	26.8		99.7	16.5	6
AB (O_2/Iron)	0.21			0.65			
1. - -		101.7	37.9		39.3	23.3	3
2. - +		190.3	37.9		88.7	23.3	3
3. + -		134.7	37.9		39.3	23.3	3
4. + +		119.7	37.9		110.7	23.3	3

Table 5.5.5: Initial and final brine density (g/mL)

Level	Initial Density g/mL			Final Density g/mL			
	P	Mean	SE (pooled)	P	Mean	SE (Pooled)	n
A: Oxygen	0.05			0.09			
stressed(-)		1.0961	0.0013		1.0947	0.0014	6
unstressed(+)		1.1003	0.0013		1.0986	0.0014	6
B: Iron	0.14			0.54			
-		1.0997	0.0013		1.0973	0.0014	6
+		1.0967	0.0013		1.0960	0.0014	6
AB (O_2/Iron)	0.98			0.89			
1. - -		1.0976	0.0018		1.0955	0.0019	3
2. - +		1.0946	0.0018		1.0940	0.0019	3
3. + -		1.1018	0.0018		1.0991	0.0019	3
4. + +		1.0989	0.0018		1.0981	0.0019	3

Table 5.5.6: Initial and final brine pH (midday)

Level	Initial pH			Final pH			n
	P	Mean	SE (Pooled)	P	Mean	SE (Pooled)	
A: Oxygen	0.96			0.4			
stressed(-)		7.88	0.02		8.08	0.03	6
unstressed(+)		7.88	0.02		8.05	0.03	6
B: Iron	0.81			0.75			
-		7.89	0.02		8.07	0.03	6
+		7.88	0.02		8.06	0.03	6
AB (O_2/Iron)	0.96			0.94			
1. - -		7.89	0.03		8.09	0.04	3
2. - +		7.88	0.03		8.07	0.04	3
3. + -		7.89	0.03		8.05	0.04	3
4. + +		7.88	0.03		8.04	0.04	3

Table 5.5.7: Percent oviparity.

Level	Initial % Oviparity			Final % Oviparity			n
	P	Mean	SE (pooled)	P	Mean	SE (pooled)	
A: Oxygen	0.51			0.1			
stressed(-)		93.3	9.75		96.5	4.2	6
unstressed(+)		83.7	9.75		85.5	4.2	6
B: Iron	0.43			0.93			
-		82.7	9.75		91.5	4.2	6
+		94.3	9.75		90.7	4.2	6
AB (O_2/Iron)	0.39			0.78			
1. - -		93.9	13.8		95.8	5.9	3
2. - +		92.7	13.8		97.1	5.9	3
3. + -		71.6	13.8		86.6	5.9	3
4. + +		95.9	13.8		84.3	5.9	3

Note: Final iron concentrations were undetectable in all tanks.

5.5.4 Discussion

With the exception of final total population of *A.franciscana*/L (Table 5.5.1) and the initial per cent oviparity (Table 5.5.7) all analysed data passed Cochran's test for homogeneity of variance. Attempts to transform the initial oviparity data and reduce variability to an acceptable level (i.e. Cochrans' test) were not successful. As the null hypotheses were retained in the ANOVA this heterogeneity did not invalidate the ANOVA (Underwood, 1981). However, it did contribute to low power. The experiment was inconclusive in determining factors responsible for switching reproductive mode. This was largely due to primarily oviparous reproduction in both the treatments designed to stimulate oviparity and their controls. The addition of iron as Fe EDTA did, however, appear to stimulate reproductive output (Tables 5.5.3 and 5.5.4) but not specifically oviparity. There was no detectable iron in any culture brine after one week. This may have utilised by the *A.franciscana* or else precipitated out even though added in a chelated form as used by Versichele and Sorgeloos (1980).

The treatments did not appear to affect the population size of animals (despite variability in final population data) or mature females (Tables 5.5.1 and 5.5.2) and brine density and pH were not significantly different between treatments at the end of the experiment, after one week (Tables 5.5.5 and 5.5.6).

5.6 Overview of Reproductive Testwork

5.6.1 General Summary

The experiments in Section 5.1 demonstrated a minimum food input of about 2 million *D.viridis* cells per animal per day, or 2 mg of ground wheat pollard per day is necessary to support reproductive output in *A.franciscana*. The removal of offspring in laboratory trials increases fecundity by increasing food supply to reproductive adults.

There was some indication that pulse feeding was an effective method of increasing fecundity in the laboratory but this method had no effect on the fecundity of *A.franciscana* in 1,000 L outdoor tank trials with either wheat pollard or *D.salina* food supply. The weekly harvesting of 20% of the adult population in 1,000 L outdoor tanks did not increase fecundity but was sustainable.

The experiments in Section 5.2 showed that selective removal of offspring via overflow screens was an effective method of preventing

population increase and allowing higher reproductive output in a less competitive environment. The ideal screen size was dependent on both the physical size of the adult *A.franciscana* and the flow rate. If animals are large and flow rates small, a screen size of 2 mm is adequate to retain the adult animals. If flow rates are high, such as in a bottom draining tank, and animals are small, a screen size no larger than 1 mm will be necessary. Maximum reproductive output was about 25 offspring per female per three day reproductive period (Figure 5.2.3).

The salinity experiments in Section 5.3 showed a variety of results but overall indicated no significant effect of brine density on either total reproductive output, or mode of reproduction from either absolute brine density or brine density-shock other than for a high percentage oviparity in dilute brine (1.024 g/mL - 1.03 g/mL).

The experiments in Section 5.4 and 5.5 were designed to identify the mechanism governing the switch from ovoviviparity to oviparity. Animal starvation and high light intensities were not effective in switching ovoviviparous animals to a viviparous reproductive mode. There was also no effect on oviparity from a combination of oxygen stress and high levels of iron in outdoor culture. These animals were, however, primarily oviparous in all treatments as was generally the case in all outdoor culture experiments and in a 0.1 ha test pond. The variability among replicates and the inconsistent changes over time in percentage oviparity meant that the experiments in Sections 5.3-5.5 had relatively low power.

5.6.2 Conclusion

The experimental work in this chapter demonstrated clearly that laboratory experiments are not always relevant to outdoor culture. An effective culture system containing primarily oviparous animals can be established outdoors in aerated brine of density about 1.10 g/mL (not critical) on a diet of *D.salina* (fed at a rate of 2,000,000 cells/animal/day) or wheat pollard (fed at 2 mg/animal/day) or in combination. Food supply can be continuous, daily, or added in a batch once weekly. Population expansion can be prevented by the use of 1 mm mesh size overflow screens which enable offspring to exit the pond whilst retaining adults.

Production rates of about 25 cysts/female/3-day period were achieved in1,000 L culture vessels containing about 20 adult *Artemia*/L. This is equivalent to about 1 g of cysts/tank/3-day period which, if sustained for a year, is eqivalent to about 1 kg/m^3/year.

CHAPTER 6: LARGE-SCALE CULTURE SYSTEM FOR *A.franciscana*

6.1 Food Supply

6.1.1 Microalgae

Mass culture of microalgae in brine as the major food source for *Artemia* is not considered economically feasible (Lavens and Sorgeloos, 1991). The experimentation undertaken in Chapter 3 has, however, enabled a reliable and economical system for the large-scale mass culture of microalgae in saltfield brine to be developed. This system was successfully tested repeatedly in 800 m^3 ponds (Plate 2, Chapter 2) throughout all seasons.

6.1.1.1 Brine Supply

An abundant supply of preconcentrated brine of a range of densities from seawater (1.025 g/mL) to salt saturated (1.215 g/mL) and extremely dense bitterns brine (1.03 g/mL) is readily available in large volumes from the Dampier Salt Limited brine concentrating ponds. Initial experimentation was undertaken with unsterilised brine resulting in complications of dominance from undesired microalgae and more serious complications from infestations of predators such as *F.salina* and *A.franciscana* necessitating the chlorination of this brine prior to use (Chapter 3, Part 5). Problems with predators are minimised by the use of high density (g/mL) brine which inhibits *F.salina*, although it still grows in brine of density 1.10 g/mL used for *D.salina* culture (Chapter 3, Part 5). Brine densities above 1.085 g/mL also prevent the hatching of *A.franciscana* cysts (Sorgeloos et al., 1986). These problems are further minimised by the use of a blend of high density (g/mL) bitterns brine and seawater which are initially free of these problem organisms. *D.salina* dominates in brine of density 1.10 g/mL or above and grows exceptionally well in combined bitterns/seawater brine which is the basic medium used by a number of commercial facilities culturing *D.salina* for production of Beta-Carotene pigment (N.Sammy[1] pers.comm.; P.Hinchcliffe[2] pers.comm). The cyst production of *A.franciscana* appears unaffected by the use of blended bitterns/seawater compared to normal brine concentrates (Chapter 5, Part 3) although this experiment had low power.

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[1] Former manager of "Wesfarmers Algal-biotechnology" at Port Gregory WA.

[2] Manager of Aquacarotene P/L at Dampier WA (1994).

The basic medium is therefore a mixture of high density bitterns brine (density 1.3 g/mL) and seawater (density 1.025 g/mL) to give a final density near 1.10 g/mL which is ideal for the optimum for cyst producing *A.franciscana* (Chapter 5, Part 3) and above the 1.085 g/mL threshold for repressing hatching of *A.franciscana* cysts collected from the brine concentrating ponds. Brines were sterilised with liquid pool chlorine (sodium hypochlorite) at a dosage rate of about 20 ppm chlorine at night to prevent the rapid dissipation of chlorine that occurs in bright sunlight. The 20 ppm level is double the minimum 10 ppm level found necessary to kill *F.salina* but gives added insurance against infestation (Chapter 3, Part 4). The pool chlorine was added into well-aerated brine which assisted mixing. The *D.salina* inoculum is added after at least three days after chlorination to allow time for toxic byproducts from chlorination to dissipate (Chapter 2, Part 4).

6.1.1.2. Brine Fertilisation

A range of commercially available fertilisers were tested and were similar in their effect on the growth response of both *T.suecica* and *Dunaliella spp.* The minimum application rate was about 0.02 g/L with all tested species in the laboratory although a good growth response of *D.salina* in outdoor culture could be achieved with only 0.01g/l (Chapter 2, Part 1). The brand of fertiliser generally used in large scale pond work was "Aquasol" due to its local availability, relatively cheap price and availability in 25 kg bags enabling a single bag to effectively fertilise an 800 m^3 pond at a dose rate of about 0.03 g/L. With this dosage rate, coupled with a recycling system described below *D.salina* populations of over two million cells per mL were sustained on a daily turnover rate of 10% for up to eight weeks on a single application of fertiliser. The dry weight per cell of *D.salina* was found to be about $6.5*10^{-11}$g (Appendix 2). This equates to a total production of 520 kg dry algae from the pond over eight weeks for a fertiliser cost of about A$50. The commercial price of dried microalgae has been assessed at >US$12/kg (Lavens and Sorgeloos, 1991).

6.1.1.3. Aeration

With the exception of a requirement for full sunlight in winter, the only critical physical condition for the growth of *D.salina* was found to be vigorous aeration (Chapter 2, Part 2). This appears related to the CO_2 requirements because the pH of unaerated brine would rapidly increase (Chapter 2, Part 2). CO_2 is critical for the growth of *D.salina* in brine (Loeblich, 1976; Vonshak,

1986). A number of aeration methods were trialed but each had specific problems described below:

6.1.1.3.1. AIR WATER LIFTS

Air water lifts were found to be impractical on a large scale due to the large number needed to be effective and the degree of labour involved in setting them up.

6.1.1.3.2. WEIGHTED AND PERFORATED POLYPIPE

Black polypipe is cheap and easily drilled. It can be weighted to the bottom and fed with air from an axial air blower to effectively aerate a pond. This is suitable for aerating the *A.franciscana* pond but a great deal of pipe-work and a very large blower was needed for it to be sustain *D.salina* culture densities for more than a few weeks.

6.1.1.3.3. "AIR/WATER WIK"

"Air/Water wik" is a porous rubber hose that acts as a giant air-stone to release a fine mist of bubbles. It is a highly effective method of aerating the brine with low energy input from a small compressor. It is not however practical with high salinity brines because salts, including insoluble gypsum, deposit and clog the pores in a few days rendering the system inoperable.

6.1.1.3.4. "AERE-O_2" UNIT

The most effective and reliable aeration system was an "Aire-O_2" unit. One of these units placed in a corner of the 20 m*40 m*1 m deep pond was sufficient to adequately aerate the whole pond and created good circulation. The "Aire-O_2" unit had the added advantage of tending to macerate contaminant *A.franciscana*. This makes these units unsuitable for *A.franciscana* ponds. "Aere-O_2" units are convenient, reliable and relatively cheap at a cost under A$1,000. A back up unit is, however, essential because a total collapse of the population of *D.salina* will occur within three or four days without strong aeration.

6.1.1.4. Screening

The continuous screening of D.salina culture brine assists greatly in the ongoing control of A.franciscana (Chapter 3, Part 4) which will inevitably contaminate the D.salina pond. The system used in test work consisted of a large self cleaning overflow screen of mesh size 500 µm supplied with about 50 m^3/h of brine delivered from a 50 mm submersible pump. The bottom of the screen was equipped with a drain to prevent the overflow of captured animals back into the brine from which they were screened. This system, coupled with a continuous

recycling of 10% per day of the culture pond brine through the adjacent A.franciscana pond, enabled populations of D.salina in excess of two million cells per mL to be maintained for about eight weeks. Large numbers of A.franciscana were by this time generally collecting on the screen and a complete collapse of the D.salina population followed within the next week. This indicates the need for several algal ponds. No problems were encountered with the brine protozoan F.salina, which caused major problems in experimental work (Chapter 3, Part 4), in the culture system described above.

6.1.1.5. Recycling

The purpose of the *D.salina* culture system is to provide food to cyst producing *A.franciscana*. Initial trials involved the pumping of *D.salina* culture brine through the adjacent *A.franciscana* pond to waste. There was a need for regular makeup of culture brine complicated by the necessity of sterilising incoming brine. A brine recycling system was therefore devised in an effort to conserve culture brine and fertilisers and to minimise problems of waste. Low salinity make-up brine (fresh water or sea-water) could then be added directly to the *A.franciscana* pond with the *A.franciscana* presumably controlling any contaminant protozoan predators (Chapter 3, Part 5). A recycling system also enables the waste CO_2 and ammonia byproducts produced by the *A.franciscana* to be utilised as nutrients by the *D.salina* which act as brine purifiers as well as a food source. The effects of the pH rise that generally occurs in *D.salina* culture brine as a result of CO_2 depletion and the drop in pH that occurs in *A.franciscana* brine, particularly when receiving wheat, tend to compensate (see Chapter 4, Part 2).

In practice, *D.salina* culture brine was delivered as food to an adjacent *A.franciscana* pond at a rate of three m^3/hour from a submersible pump. This elevated the level of culture brine in the *A.franciscana* pond and enabled a gravity driven return flow through a series of screens to prevent the input of *A.franciscana* nauplii or adults into the *D.salina* pond. The screens were kept clear with air curtains with sizes determined from the experiments described in Chapter 5, Part 2 and were essential in the control of *A.franciscana* populations as described below. The primary screen was a 1 mm wedge-bar screen which prevented the throughput of large adult *A.franciscana*. Culture brine containing *A.franciscana* cysts and nauplii was then screened through a 150 µm stainless steel mesh screen. The brine between the two screens, containing large numbers of *A.franciscana* cysts, was separately pumped over a 150 µm wedge-bar screen for the collection of cysts.

6.1.2. Wheat Pollard

Wheat pollard can be purchased for under A$10 for a 45 kg bag or about 25 c/kg. When crushed to a fine flour of particle size less than 50 m, in a large ball mill, it was a suitable food for *A.franciscana* with an optimum feeding level of 1 mg/animal/day (Chapter 4, Part 2) although some *Dunaliella spp.* appeared necessary for healthy animals. 1 kg of pollard produced over 1 kg of *A.franciscana* in one experiment (Chapter 4, Part 2). In 1991 the annual world demand for *A.franciscana* biomass was over 3,000 tonnes with prices above U.S.$20./kg (Lavens and Sorgeloos, 1991). Experiments undertaken in both Chapters 4 and 5 showed that similar populations of *A.franciscana* could be sustained by daily feeding or once weekly feeding at the same level, presumably due to secondary nutrition from bacteria feeding on decomposing, uneaten wheat. This implied that a continuous feeding system for wheat pollard was not essential and that a more practical daily batch feeding system was adequate for pond cultured *A.franciscana*.

Prepared wheat pollard is heavy and difficult to keep in suspension, even in hypersaline brine. 20 kg of wheat pollard was added daily to a tank containing about 500 L of culture brine and kept suspended with a recycling submersible pump. This mixture was then siphoned into a well-aerated *A.franciscana* Pond over a four hour period to ensure an even distribution in the pond.

6.2 A.franciscana *Culture System*

The optimal culture conditions for *A.franciscana* have been well reported in the literature (Chapter 1, Part 1). The specific culture conditions for an applied large-scale culture facility had to be developed over time with consideration being given to a diverse range of factors including optimisation of food supply; optimisation of physical and chemical conditions for both *A.franciscana* growth and *A.franciscana* reproduction and; compromising on the optimum conditions for the growth of *D.salina* in an integrally linked system.

6.2.1 Brine Density (g/mL)

A.franciscana are tolerant of a very wide range of physical and chemical conditions which have been well studied (Persoone and Sorgeloos, 1980). The critical aspect for intensive culture appears to be oxygen level Sorgeloos et al., 1986). The solubility of oxygen decreases with increasing salinity (Appendix 1). It is therefore appropriate to maintain salinities at relatively low levels. The

reproductive response of *A.franciscana* to brine density and brine density-shock is addressed in Chapter 4.3. This showed that salinity is not a critical factor in influencing either mode of reproduction or total reproductive output in the brine density range 1.08-1.16 g/mL. The quality of produced cysts must, however, also be considered and cysts produced from animals in higher salinities have superior hatching percentage (Lavens and Sorgeloos, 1987).The degree of dehydration of produced cysts is also dependent on salinity and the rapidity of deterioration of produced cysts is dependent on the degree of dehydration (Lavens and Sorgeloos, 1987). The other problem of low salinities is that cysts can hatch in brine densities under 1.085 g/mL (test work on Dampier strain *A.franciscana*). When considered in conjunction with the conditions required for the mass culture of *D.salina* in the linked microalgae food pond (see above) a brine density of 1.10 g/mL is close to ideal. This gives scope for evaporative concentration up to a brine density of about 1.14 g/mL without causing major problems for either pond but in practice the frequent addition of seawater keeps brine densities close to 1.10 g/mL.

6.2.2 Aeration

The provision of an efficient aeration system is critical for the mass culture of *A.franciscana*, particularly in summer when brine temperatures can reach 35^{o}C. The aeration system oxygenates the brine and allows for the even distribution of food, particularly wheat pollard within the pond. A number of aeration systems, similar to those tested for the *D.salina* pond (see above) were trialed. A system of high turbulence from relatively large bubbles was finally developed. This consisted of a number lengths of 32 mm black polypipe drilled with 2 mm holes at 1 m intervals running off a central 90 mm delivery pipe, also drilled. This aeration web was weighted to the floor of the pond with heavy bricks and fed with air delivered from a large axial air blower. The depth of brine in the pond needed to be at least 1 m and preferably 1.5 m for good mixing with this system. Shallower depths did not allow sufficient turbulence.

6.2.3 Feeding System

The growth response of *A.franciscana* to food supply is addressed in Chapter 4 and the reproductive response in Chapter 5. In practice, growth and reproduction occur within a single pond. The optimum feeding level for growth in *A.franciscana* is between 1 million and 2 million *D.salina* cells/day giving a growth efficiency (dry weight/dry weight) of about 25% (Chapter 4, Part 2). The upper

range of this optimal feeding level was found necessary for good reproductive output (Chapter 5, Part 1). This means that reproductively mature animals should each receive 2 million *D.salina* cells per day or the equivalent mix of *D.salina* and wheat pollard. Wheat pollard is not as effective as a diet with 1 mg of wheat being equivalent to about 1 million *D.salina* cells (Chapter 3, Part 2). Wheat pollard is an effective food on it's own but some *D.salina* (about 200,000 cells/animal/day) was needed for the animals to attain good colour (Chapter 3, Part 2).

The food delivery system for both *D.salina* and wheat pollard is described above. The delivery of 3,000 L per hour of *D.salina* culture containing two million cells per mL will optimally feed about 70 million animals at a rate of two million cells per animal per day. The provision of 20 kg of wheat pollard will support an additional ten million animals at 2 mg pollard per animal per day to give a final population in the 800 m^3 *A.franciscana* culture pond of about 100 animals/L. In theory the wheat pollard input could be increased by an order of magnitude but brine quality problems in terms of low pH and low levels of dissolved oxygen became apparent with pollard feeding rates in excess of about 40 kg per day. The other constraint with wheat pollard was the time taken to physically mill the wheat pollard in the ball mill. A batch of 40 kg of pollard required tumbling for about six hours prior to use to achieve a fine flour with most particles below about 10 µm and largest particles below 50 µm. However, more efficient grinding systems could be developed.

6.2.4 Selective Screening System

A selective screening system was used in conjunction with the brine recycling system used above. This was found to be necessary in order to restrict the population expansion of *A.franciscana* and to ensure broodstock received optimum levels of food for reproductive output which would peak at 25 offspring per female per three day reproductive period (Chapter 5, Part 2). In the absence of screening the *A.franciscana* population will expand to a point where each adult animal receives about 500,000 *D.salina* cells per day (Chapter 4, Part 1) and reproductive output is very low (Chapter 5, Parts 1 and 2). The mode of reproduction in outdoor experimentation and in the test ponds tended to be primarily oviparous (Chapter 5, Part 5).

The main considerations in developing a screening system were as follows:

6.2.4.1. Mesh Size

A 1 mm primary screen was needed to retain adults (Chapter 5, Part 2). In practice a stainless steel wedge-bar screen was used.

A 150 µm screen was needed as a secondary screen to prevent the input of any *A.franciscana* cysts or nauplii into the *D.salina* pond.

6.2.4.2. Screen Dimensions

The necessary physical dimensions of the screens are dependent on the flow rate; amount of suspended material suspended in the brine; and the effectiveness of the air curtain, or similar, to keep the screen clean. A screen of dimensions 1.5 m X 1.5 m worked effectively for many weeks in the developed operational system with major scrubbing undertaken every two weeks.

6.2.4.3. Sealing of Screens

The major practical problem in the whole system was the physical sealing of the screens to the faces of the concrete weir. High pressures build up on the *A.franciscana* Pond side of the screen with differential heights and the screens must be well secured and sealed. The screening material must also be strong and reinforced to prevent it tearing under the pressure. Any leaks in the secondary screen will allow *A.franciscana* to enter the *D.salina* pond.

6.2.5 Cyst Harvesting Techniques

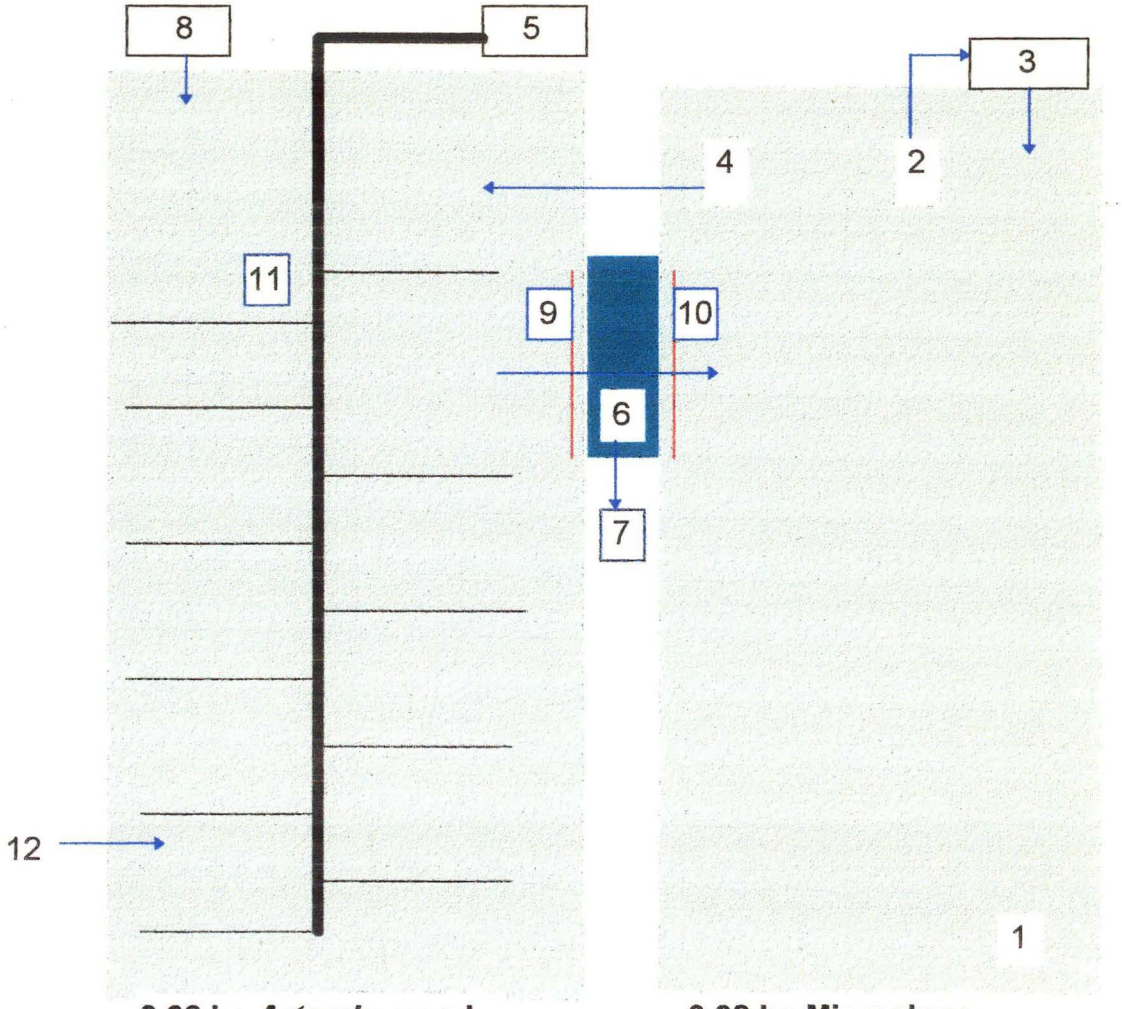
It took about six weeks from first flooding and inoculation before the *A.franciscana* populations in the *A.franciscana* Pond reached their peak populations with each adult female producing about 25 cysts per three day reproductive period. A female population of 50,000/m^3 in an 800 m^3 pond is therefore producing $2*10^9$ cysts per 3 day period. Cysts weigh about 300,000 per gram(obtained by accurately weighing out 0.1 g of dry cysts, thoroughly mixing in 1 L of brine and counting the number suspended in consecutive 1 mL samples) equating to a daily production for the pond of about 2 kg of cysts although in practice only 1 kg per day could be harvested. Some were collected from the sump between the two screens onto a 150 µm self cleaning wedge-bar screen; Some, adhered to the wooden sides of the pond, particularly in the leeward corner and were collected physically by scraping and; a large proportion were

present in foam in the leeward corner of the pond and were skimmed after collapsing the foam with a jet of water. These labour intensive methods were used to harvest 20 kg of cysts over a 20 day period which is estimated to be about half of the total production. The majority of losses are suspected to be via wind blown foam.

The tendency for the supply of *D.salina* food from the microalgae pond to collapse between six and eight weeks necessitated the installation of a backup *D.salina* pond. This second pond, operated two weeks out of phase, ensured a reliable supply of microalgae food was always available. It consistently took about two weeks from first inoculation for the *D.salina* population to peak at about two million cells/mL at all times of the year.

6.2.6 Cyst Processing and Storage

Collected cysts were placed in high density (>1.18 g/mL) brine. The buoyant cysts float and heavy wheat pollard contamination sank. They were then rinsed through a 1 mm screen to remove insects and collected on 112 µm nylon mesh (Swiss Screen) and excess moisture was squeezed from them by hand. The cysts were then rinsed with fresh water to remove salt and squeezed to as dry as possible. They were then sun dried and rubbed through a rigid 500 µm metal screen to break up the hard cakes and the dried product vacuum sealed in plastic bags. Sun drying is not recommended (Sorgeloos et al., 1986) but was used in the absence of no immediate practical alternative. For a full-scale production facility one of the various air and tumble drying techniques recommended by Sorgeloos et al. 1986 would be used.



Codes

1. 2HP "Aere-O2" Unit
2. 50 mm submersible pump
3. 500 micron wedge-bar screen
4. 32 mm submersible pump feeding *Artemia* pond
5. Large air blower
6. Submersible pump to feed cyst harvester
7. 150 micron screen
8. Pollard feeder
9. 1 mm primary screen
10. 150 micron screen
11. Air manifold
12. Make-up brine

Figure 6.1: Large-scale culture system for *A.franciscana* cyst production.

CHAPTER 7: GENERAL SUMMARY AND DISCUSSION

7.1 Introduction

An operational cyst producing facility (Chapter 6) was created by integrating the experimental findings presented in Chapters 3, 4 and 5. There is no record in the literature of similar large-scale intensive systems for the production of *Artemia* cysts although intensive small-scale systems for biomass production (Lavens and Sorgeloos 1991, Sorgeloos et al., 1986) and semi-intensive culturing in fertilised ponds (Tackaert and Sorgeloos, 1991) are well documented.

Chapter 7 is a general summary and discussion section highlighting aspects of original research and areas warranting further investigation.

7.2 Microalgae Mass Culture

Although the commercial production of live microalgae as food for *Artemia* in intensive culture systems is not considered economically feasible (Ali and Brendonck, 1995, Lavens and Sorgeloos, 1991) maintenance of a healthy phytoplankton population is considered one of the important keys for the successful *Artemia* production in ponds (Tackaert and Sorgeloos, 1991). The current prices of commonly used animal feeds (e.g. grains and soymeal) are about one-tenth the cost of the production of algae in the small operating plants currently in use but the scale of production has the most decisive effect on per unit production costs (Richmond, 1991b). The economic aspects of mass culturing brine microalgae, which are the natural food of *Artemia*, in large ponds as food for *Artemia* has not, however, been well researched. Much of the experimental data presented in Chapter 3 was from baseline experiments. This was presented in four sections and ultimately enabled the production of 520 kg (dry weight) of *D.salina* algae to be produced in a 0.08 ha pond over an eight week period for a cost of about A$50 worth of fertiliser and aeration costs (Chapter 6). This is based on a single 25 kg bag of Aquasol liquid fertiliser added to 800 m^3 of aerated brine (Aere-O2 unit) pond resulting a standing crop of two million *D.salina* cells/mL diluted 10%/day for eight weeks. The dry weight per cell of *D.salina* was found to be about $6.5x10^{-11}$g (Appendix 2).

7.2.1 Determination of Fertiliser Type and Level

The determination of effective fertilisation strategies was the initial focus of microalgae test work. A series of laboratory experiments were undertaken trialing the growth response of *D.salina* and *T.suecica* to various concentrations of complete inorganic fertiliser and equivalent amounts of phosphate and nitrate in sterile saltfield brine. *D.viridis* would

not grow in the laboratory. More complete fertilisers (e.g. Aquasol) gave a very good microalgal growth response and may contain some essential ingredient (not identified) apart from these two vital macronutrients not provided by the brine. *D.salina* was much more productive than *T.suecica* in terms of cell numbers and displayed highest productivity at dosage rates >0.02 g/L. The Dampier strain of *T.suecica* had much larger cells (up to 50 µm diameter) than *D.salina* (generally between 5 and 10 µm diameter). There was no significant difference in growth response to various brands of fertiliser. Later experiments (Chapter 2.2) demonstrated that in an outdoor situation a fertilisation rate of 0.01 g/L was sufficient to maximise the growth response of *D.viridis* and *D.salina*.

The fertilisation experiments were undertaken from a practical viewpoint to develop an efficient and cost-effective brine fertilisation technique for large-scale outdoor culture. A fertilisation level of 0.02 g/L or even 0.01 g/L is an order of magnitude less than the 1 g/L concentration of macronutrients (excluding those abundant in brine) recommended by Borowitzka (1974) in standard culture media for *Dunaliella*. Complete inorganic fertilisers are readily available and relatively cheap. There is great scope for similar experiments into the dose-effect relationships of different inorganic fertilisers on specific types of microalgae. This is also the conclusion of De Pauw and Persoone (1988), in their review of microalgae for mariculture.

7.2.2 Optimising Culture Conditions for Brine Microalgae

Experimentation to determine culture conditions to maximise productivity (cells/volume/day) of *T.suecica, D.salina* and *D.viridis* in brine was undertaken in Chapter 3.3. Brine density g/mL (Richmond and Becker, 1986), light intensity (Richmond, 1986a) and degree of turbulence (Richmond and Becker, 1986) are three easily manipulated factors known to affect the productivity of microalgae although more study into the effects of these variables is needed (De Pauw and Persoone, 1988).

7.2.2.1 Brine Density (g/mL)

T.suecica was completely inhibited by brine densities above 1.10 g/mL (Experiment 3.3.1) in the laboratory. This is close to the ideal brine density (g/mL) for rearing cyst producing *A.franciscana* (Chapter 6) and complicates the use of this species in an outdoor culture situation where evaporative losses could cause brine density increases. *D.salina* on the other hand grew well at all brine densities within a trialed brine density range of 1.06-1.09 g/mL (Experiment 3.3.2) and will tolerate brine densities up to salt saturation (Borowitzka, 1974; Borowitzka and Borowitzka, 1989) so population

collapse as a result of salinity increase is unlikely. Problems with predatory protozoans and undesired competitor algae such as diatoms and benthic Cyanobacteria are also greater at lower brine density (discussed fully in Section 3.4). The option of controlling these problem organisms by elevating salinity is possible with *D.salina* but not with *T.suecica* culture.

The physiological effects of salinity on *Dunaliella* have been well researched (Borowitzka, 1974) but there is great scope for experimentation into the use of salinity to control dominance of desirable microalgae species in mixed populations of microalgae.

7.2.2.2 Aeration and Shading

Outdoor experiments to test the effect of aeration and shading in 1,000 L tanks were conducted under winter (Experiment 3.3.2) and summer conditions (Experiments 3.3.3 to 3.3.4). Vigorous aeration was critical to sustain growth of both *D.viridis* and *D.salina* in brine fertilised with 0.01 g of complete inorganic fertiliser under both winter and summer conditions. The use of 80% shadecloth in winter (maximum intensities at noon of about 2,000 lux) inhibited substantial growth of any algae including *D.viridis* which grew well under full winter sun conditions (17,500 lux) (Experiment 3.3.2). Light intensities of about 2,000 lux are equivalent to the light intensities experienced in the environmental chamber used for indoor algae culture and *D.viridis* would not grow in this chamber. *D.viridis* would grow under shadecloth in summer (7,000 lux at noon) (Experiment 3.3.3) but grew equally well under full sun conditions (23,000 lux). *D.salina* also grew well under full summer sun conditions (Experiment 3.3.4). The ability to mass culture microalgae without shading has significant economic benefits as does the high yield (up to 4,000 $x10^3$ *D.viridis* and over $1,000x10^3$ *D.salina* cells/mL equivalent to 0.65 and 2.6 g/mL dry weight/mL) achieved with only 0.01 g of fertiliser/L.

The pH became greatly elevated in tanks without vigorous aeration (Table 3.3.12), possibly due to a depletion of CO_2 from the brine column. The relative concentrations of the inorganic carbon species determine the pH and in turn are determined by the pH (Kaplan et al., 1986). The removal of bicarbonate and CO_2 from brine will elevate the pH. Benthic cyanobacteria were abundant in tanks with little or no aeration. These tanks were receiving less atmospheric CO_2 than the vigorously aerated tanks. The removal of CO_2 from the brine increases the pH and causes CO_3^{2-} to become the major inorganic carbon species. This may well have given the Cyanobacteria an advantage over the *D.salina* which grew well initially without aeration due to the ability of cyanobacteria to utilise bicarbonate as a carbon source (Kaplan et al., 1986). Attempts to manipulate brine pH in the unbuffered cultures were not successful.

This indicates that direct pH adjustment of brines is difficult without the use of buffers or regular inputs of acid.

The necessity of vigorous aeration to sustain mass cultures of *Dunaliella spp.* is a significant expense for large scale culture. The necessity of aeration appears to be linked to the maintenance of constant pH (about 8.0) and CO_2 level. It is possible that less aeration would be required if CO_2 could be directly injected into the culture. Olaizola et al. (1991) found that periodic injections of CO_2 were necessary to sustain long term *D.salina* cultures. It is also possible that constant addition of acid would have a similar effect. Both these aspects are worthy of investigation and are not covered in the thesis. Another aspect worthy of serious study is the optimisation of factors such as brine depth and pond contours for maximum mixing.

7.2.3 Maximum Sustainable Yields

The growth response over time of populations of *D.salina*, *D.viridis* and *T.suecica* in the experiments described in Parts 1 and 2 of Chapter 3 gave some indication of productivity under a range of conditions. The sustainable yield of laboratory cultured *D.salina* (Section 3.2, Experiment 3.2.2) was as high as 25%, equivalent to (500,000 cells per mL per day) but was much less in outdoor cultures which were influenced by infestations of *F.salina* and competition with benthic algae. Van Auken et al. (1973) achieved a ten hour doubling time for Great Salt Lake *Dunaliella* and the maximum recorded productivity of *D.salina* at 60 g Dry weight/m^2/day (Ben-Amotz, 1980) is extremely high.

Experiments (3.4.1 and 3.4.2) were conducted to determine maximum sustainable yields of both *D.salina* and *D.viridis* in outdoor culture but both were confounded with protozoan *(F.salina)* infestation. The slow but definite increase in *D.salina* population up to Day 17 at a daily turnover of 40% in Experiment 3.4.2 did, however, indicate that it is possible to sustain cultures at this level of dilution (Figure 3.4.1, Table 3.4.6) provided that predators such as *F.salina* could be controlled. However, algal production estimates for an 800 m^3 pond were based on only 10% per day.

The productivity of mass cultured *A.franciscana* will largely depend on the productivity of their brine microalgae food supply. Maximum sustainable yield is a measure of productivity and an indication of the success at integrating all factors necessary for high yield microalgae culture. It is important to know the maximum sustainable yield in order to determine food supply and culture volumes to be transferred. This in turn will impact directly on the design of the *A.franciscana* culture system. Maximum sustainable yield is a measure of productivity and

while it can be used to bench-mark one facility against another it is a site specific factor.

7.2.4 Remedies to Problems

The development of remedies to problems such protozoan infestation, *A.franciscana* contamination, and dominance of undesired microalgae species encountered during in the course of experimentation and in pilot pond trials was critical to the establishment of a successful project. Control methods were developed largely from first principles using a combination of laboratory test work and trials in large scale culture ponds (Chapter 6). Much of the work was undertaken on a trial and error rather than detailed experimental basis and Chapter 3.4 is largely descriptive.

7.2.4.1 A.franciscana *Contamination*

The elimination and control of *A.franciscana* from the microalgae ponds was an ongoing practical problem. The strategy was to begin with uncontaminated culture brine (sterilised with >10 ppm chlorine) and to use extreme care to avoid mixing any *A.franciscana* culture brine with the adjacent microalgae culture brine. The use of brine of density >1.085 g/mL will prevent any hatching of windblown cysts (Sorgeloos, 1986). The complete sealing of secondary 150 µm screens separating the *A.franciscana* and microalgae ponds (Chapter 6) was found to be very difficult. Low levels of contamination can be controlled for several weeks using recycling pumps delivering culture brine over a 500 µm wedge bar screen and the use of an impellor driven "Aere-O_2" unit for aeration also assists because it macerates the soft bodied *A.franciscana*. The complete control of *A.franciscana* was not achieved but the above methods generally enabled the microalgae pond to operate from six to eight weeks before being cleared by *A.franciscana*.

7.2.4.2 F.salina

Five experiments were undertaken to identify effective control methods for *F.salina* in Chapter 3.5. Protozoan contamination, which can devastate cultures in less than 24 hours, despite the use of sterilisation and ultrafiltration techniques, is the most common problem for commercial microalgae farms (De Pauw and Persoone, 1988). The following work, which was preliminary from a scientific viewpoint, has wide commercial relevance.

Diatomaceous Earth Filtration (Experiment 3.5.1) was not successful and was considered practically difficult to apply effectively to large volumes of brine as it necessary to filter out every *F.salina* cell. It is

still, however, a potentially good control method that could perhaps be developed.

In Experiment 3.5.2, a concentration of 10 ppm chlorine was sufficient to kill all *F.salina*. This method is easy to apply to large volumes of culture and in fact was the method used in the pilot trial described in Chapter 6.

The thermal bioassay conducted in Experiment 3.5.3 demonstrated that temperatures >45^{o} C would kill all *F.salina*. This option was not applied but could be considered in areas with excess heat production such as thermal power stations.

High brine density (g/mL) (>1.15 g/mL) inhibited *F.salina* in Experiment 3.5.4 but was not considered a practical option for their control. It was apparent, however, that problems with *Fabrea* would be minimised by operating ponds with as high a brine density as possible.

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A.franciscana were effective in eliminating *F.salina* from the brine column in Experiment 3.5.5 (Table 3.5.2). This suggested that fresh brine inputs should be added to the *Artemia* pond rather than the microalgae pond in an interconnected system similar to that described in Chapter 6.

7.2.4.3 Control of Competitor Microalgae

The experiments described in Chapter 3.2 demonstrated the importance of optimising physical conditions in order to maintain cultures of microalgae. If conditions were not optimised undesired competitors, particularly benthic diatoms and cyanobacteria tended to dominate. As mentioned previously chlorination was found to be the most practical and effective method of initially eliminating all competitor microalgae from the brine.

7.2.4.4 Failed Experiments

In addition to the experiments described in Chapter 3, a large number of unreported microalgae experiments, particularly *T.suecica* experiments were commenced and abandoned for various reasons. These are listed in Appendix 6.

7.3 The Growth Response of A.franciscana *to Food Level*

The growth response of *A.franciscana* to food level is the subject of Chapter 4 and presented in two parts.

7.3.1 The Growth, Survival and Food Conversion for *A.franciscana* Fed Mass Cultured *Dunaliella spp.* in Field Brine (Chapter 4.1)

The growth response of *Artemia* to a range of microalgae foods is well documented in the literature (Mason, 1963; Reeve, 1963a,b,c; Johnson, 1980; Brune and Anderson, 1989, 1984; Rowsowski 1989) although no one has previously used brine microalgae as a food source.

Four experiments were undertaken. In Experiment 4.1 the growth response of communal *A.franciscana* to food ration of *D.viridis* was investigated. It was apparent that maximum growth rates had not been achieved but it was also apparent that the lowest rations (100,000 and 200,000 cells/animal/day) were too low for animals to reach sexual maturity and they ultimately starved. Growth efficiencies could not be determined because it was not known what proportion of the food was consumed by individual animals within the communal beakers. Food conversion efficiency is often based on groups of animals rather than individuals. The main innovation in methodology was the temporary stranding of animals on a cavity slide under a dissecting microscope for measurement. This method made accurate measurement of larger animals difficult because they would stretch and contract but was considered less stressful to the animals than the standard narcotisation with chloroform technique used by Gilchrist (1959).

Experiment 4.2 examined the growth response and efficiency of solitary *A.franciscana* in relation to food ration of *D.salina*. The upper range of food ration was extended in an attempt to achieve maximum growth rates. Maximum growth rates were achieved with four million *D.viridis* cells/animal/day and there was a pattern of decreasing growth efficiency with increased ration. The overall growth efficiencies were similar to those obtained by Mason (1963) using *D.tertiolecta* as a food source. The highest growth efficiencies were achieved at a ration of 500,000 cells/animal/day and it was apparent that this ration was insufficient for *A.franciscana* to reach full maturity. Growth efficiencies at a feeding level between one and two million cells/animal/day gave efficiencies of about 25% and enabled full maturity to be reached. This range was considered optimal.

Experiment 4.3 compared the growth response of *A.franciscana* in solitary and communal culture to food ration of *D.salina*. This experiment was primarily undertaken to assess the effect of competition on growth rates and to determine the relevance of the growth efficiency data of Experiment 4.2 to communal culture. There was no significant difference in animal size attributable to culture conditions indicating that the efficiency data from Experiment 4.2 is valid for communal culture. The

most significant finding from a scientific viewpoint is that variability in size in solitary culture was high and similar to communal culture indicating intrinsic (i.e. genetic) variability and contradicting the findings of Brune and Anderson (1989) who used a mathematical model to predict high size variation among competing animals and a smaller variation in size when competition was reduced. The mean communal feeding levels of one million and two million cells/animal/day may not, however, have caused severe competition. Despite this, the ecological implication is that some precocious individuals can quickly become mature after a rain event hatches dormant cysts. This would maximise the chances of some reproduction prior to the pool drying up if no follow up rain eventuated. This aspect is worthy of further study from an ecological viewpoint. It also has implications for communal culture in ensuring some recruitment in a system equipped with an efficient offspring removal system such as that developed for commercial culture at Dampier Salt (Chapter 5.2 and Chapter 6).

Experiment 4.4 examined the population response of various populations of communal *A.franciscana* to a constant food ration of *D.salina.* This experiment was designed to determine whether constant input of food at specific levels resulted in population fluctuations. Significant changes in population did occur and it was concluded that in an uncontrolled population situation the population would expand to a level where each individual was receiving 500,000 cell/day shown in Experiment 4.2 to be too low to enable full maturity to be reached. There is great scope for in depth studies into population responses to food supply and the ecological implications. From a commercial cyst production viewpoint it would be necessary to control population expansion.

7.3.2 The Growth Response of *A.franciscana* to Wheat Pollard and Mixed Pollard *D.salina* Diet (Chapter 4.2).

A great number of inert foods have been trialed successfully as food for *Artemia* (D'Agostino and Provasoli, 1968; Viera, 1987; Douillet, 1987) but wheat pollard, which is very cheap and readily available in Australia has not been.

Four experiments were conducted to determine the growth response of *A.franciscana* to finely ground wheat pollard flour and mixed pollard/microalgae diet.

Experiment 4.5 was designed to test the growth response of solitary *A.franciscana* to wheat pollard diet and proved that wheat pollard was a good food for *A.franciscana* in the laboratory.

The growth efficiency and population response of communal *A.franciscana* In 500 mL Beakers to *D.salina* and Mixed *D.salina*/Wheat

Pollard Diet was investigated in Experiment 4.6. The growth efficiency (dry weight animal/dry weight food) was about 20% for *D.salina* and 5-6% for pollard supplemented diet. There was an indication that a specific ration enabled a maximum individual animal size to be achieved and further growth was only possible with population decline. This is an interesting and complex aspect warranting further study.

Experiment 4.7 was designed to test the growth efficiency and population response of communal *A.franciscana* in 1,000 L outdoor tanks to *D.salina* and mixed *D.salina*/Wheat Pollard diet. The wet weight of *A.franciscana* in tanks receiving wheat was significantly higher than in tanks without wheat confirming the findings of the laboratory Experiments 4.5 and 4.6 that wheat pollard is a suitable food for *A.franciscana.* It also confirmed its suitability for large scale outdoor culture despite the fact that batch feeding resulted in deposits of black decaying detritus on the bottom of tanks receiving wheat. This problem could be minimised by the development of an effective continuous feeding system and although some practical investigation was undertaken into this it remains an area for further study.

Experiment 4.8 was designed to investigate the growth efficiency and population response of communal *A.franciscana* in 1,000 L outdoor tanks to wheat pollard with and without *D.salina* supplements. The addition of *D.salina* amounting to 5% on a dry weight basis doubled the wet weight of *A.franciscana.* The food conversion efficiency (ww *Artemia*/dw food) was close to one for tanks receiving supplementary live microalgae and about 0.5 for tanks only receiving pollard. Conversion efficiencies of one have been achieved using micronised rice bran in both batch (Bossuyt and Sorgeloos, 1981) and flow through (Brisset et al., 1982) culture without microalgae supplements.

7.4 Factors Affecting the Reproductive Response of the Dampier Strain of A.franciscana (Chapter 5).

The investigation of factors affecting the reproductive response of Dampier strain *A.franciscana* was a key study area both from a practical applied and a scientific viewpoint. Despite the wealth of published information on *Artemia* reproduction much of the experimentation in this section was first principles research with very little published information to refer to. Chapter 5 was presented in five parts.

7.4.1 The reproductive response of *A.franciscana* to food supply.

Five experiments were conducted to determine the reproductive response of A.franciscana to various diets. *Artemia spp.* receiving high levels of food are known to have increased fecundity (Lenz, 1980; Lavens and Sorgeloos, 1987) although starving animals possibly

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produce better quality cysts than well-fed ones (Lavens and Sorgeloos, 1987).

In Experiment 5.1 the reproductive response of laboratory cultured *A.franciscana* to *D.viridis* mass cultured in saltfield brine was investigated. Spawnings occurred every three days and it was concluded that a minimum feeding level of two million *D.viridis* cells/animal/day was needed to support reproductive output. This is the upper end of the feeding level found to be optimal for *A.franciscana* growth in Experiment 4.2.

In Experiment 5.2 the reproductive response of laboratory cultured *A.franciscana* to ground wheat pollard and the effect of competition and pulse feeding on this response was investigated. The optimum quantity of wheat pollard for both total reproductive growth and animal survival was 2 mg per animal per day. Higher feeding levels retarded growth in the 500 mL unaerated beakers in the laboratory. The removal of offspring and the subsequent decrease in competition for food resulted in significantly increased fecundity. This is consistent with the findings of Experiment 5.1 and studies on the reproductive response of *Artemia* in a number of natural systems where reproductive output correlated significantly with food level (Lenz and Browne, 1991).

In Experiment 5.3 the effect of pulse feeding and animal harvesting on *A.franciscana* populations and their production of cysts was investigated in 1,000 L tank culture. Neither animal harvesting or pulse feeding was effective in limiting the mean population of *A.franciscana* in 1,000 L tanks. There was also no significant difference in the total wet weight of animals harvested from the tanks at the end of the experiment indicating that the animals in different treatments were of similar size (Table 5.1.5). Although the mean number of suspended cysts was very similar between treatments data were highly variable and not useful. Attempts at increasing reproductive output via a reduction in intraspecific competition have not been seen in the literature. The weekly removal of 20% of the population >1 mm on a weekly basis appeared to be sustainable with no significant decrease in wet weight harvest from the second to the fourth week .

Experiment 5.4 was designed to test the reproductive stimulus of pulses of food fed to starving animals. The high productivity of *D.salina* within the tanks, however, ensured animals in both treatments were at all times well fed. It is not surprising, therefore, that no significant differences in animal populations or reproductive output were found.

Experiment 5.4 was repeated in Experiment 5.5 with half the brine turnover. The *A.franciscana* in this experiment effectively cleared the brine in all tanks but feeding strategy had no significant effect on animal population sizes; reproductive output, or percent oviparity with animals remaining basically in oviparous mode throughout the experiment.

7.4.2 The use of screens to minimise competition in communal *A.franciscana* culture (Chapter 5.2).

Experiments 5.3-5.5 failed to identify a strategy that effectively prevented *A.franciscana* population expansion and maximised cyst production. The next step was to investigate various selective screening techniques. As with the experiments in Chapter 5.1 there were no reports in the literature on similar experiments.

In Experiment 5.6, 500 m and 700 m screens were used in an attempt to limit *A.franciscana* population growth in 1,000 L outdoor tanks. The fine mesh used as a secondary screen in this experiment clogged quickly with detritus and prevented through flow of brine. This may have contributed to the lack of effectiveness in limiting population expansion in any treatment and of the close similarities in final populations of animals at the end of the trial.

The effect of 0.4 mm and 2 mm mesh screens on *A.franciscana* populations in 1,000 L outdoor tanks was investigated in Experiment 5.7. The 2 mm screens effectively retained mature females but allowed offspring to pass through resulting in significantly reduced overall populations of *A.franciscana* in these tanks. Reproduction data was variable but there did appear to be some increase in reproductive output in tanks with reduced overall populations.

In Experiment 5.8 the use of 0.4 mm and 1.6 mm screens to restrict *A.franciscana* population expansion in 1,000 L outdoor tanks was investigated. The total population of animals in tanks equipped with 1.6 mm screens had reduced greatly after one week but the fecund female population was not significantly different confirming that a 1.6 mm screen is also an effective option for retaining mature *A.franciscana*.

In Experiment 5.9 the use of 0.4 mm and 1.6 mm screens with and without brine recycling on *A.franciscana* population growth and reproductive output in 1,000 L outdoor tanks was investigated.The experiment confirmed that a screen size of 1.6 mm was sufficient to retain adult *A.franciscana* under conditions of gravity flow. Active pumping, however, seems to draw the adults through this sized screen. The loss of food through an overflow system could possibly be minimised by overflowing brine after most food has been consumed, or by recycling the overflowed brine back through the tanks.

In Experiment 5.10 the population and reproductive response of *A. franciscana* to selective removal of offspring via overflow screens was investigated. The experiment was complicated by changes in the concentration of *D.salina* food contained within the 500 L daily added brine during the course of the experiment. Despite this problem, the experiment confirmed that a 1.6 mm screen (in this case bottom draining) will retain most mature *A.franciscana* (there were some losses) and prevent population expansion by effectively removing offspring. This

in turn increased reproductive output to a peak of 25 per female per three day reproductive period. This figure is close to the reproductive maxima/female achieved in previous experiments (Experiment 5.4) and in a 0.1 ha experimental pond (Chapter 6).

7.4.3 The reproductive response of *A.franciscana* to salinity.

It is widely reported that *Artemia spp.* are ovoviviparous in low salinity ponds and oviparous in high salinity ponds (Davis, 1980; Sorgeloos et al., 1986; Brown et al., 1991; Tackaert and Sorgeloos, 1991) in which cysts accumulate. Berthelemy-Okazaki and Hedgecock (1987) on the other hand report that oviparity decreases with increasing salinities. Four experiments were undertaken to determine the reproductive response of A.franciscana to salinity.

In Experiment 5.11 the reproductive response of *A.franciscana* to brine density was investigated and the trend of decreasing oviparity with increasing salinity was consistent with the findings of Berthelemy-Okazaki and Hedgecock (1987) and of Williams and Mitchell (1992) in their study with parthenogenetic South African *Artemia*. The maximum reproductive response in the 1.08 g/mL (107.4 ppt salinity) density treatment was at the lower end of the 100 to 170 ppt salinity range found to maximise *Artemia* reproductive output in a study by Wear and Haslett (1987) for Lake Grassmere *Artemia* when brine temperature was between 20^{o}C and 28^{o}C. It is slightly above the 75 ppt salinity that maximised reproductive response with parthenogenetic South African *Artemia* in the study by Williams and Mitchell (1992).

In Experiment 5.12 the reproductive response of *A.franciscana* to brine density and brine density-shock was investigated and failed to conclusively confirm the results of Experiment 5.11 due to variable data but trends were consistent with the earlier experiment.

In Experiment 5.13 the reproductive response of *A.franciscana* to salinity-shock was investigated and was not significantly different from control animals.

In Experiment 5.14 the effect of brine quality on *A.franciscana* reproduction was investigated and brine quality had little effect on reproductive output or percent oviparity at a brine density of 1.10g/mL. This indicates that *A.franciscana* culture brines can be derived from seawater to which either salt or bitterns has been added as well as from normal seawater concentrate brine and adds great flexibility to culture brine options.

The above experiments indicate that salinities do not have a marked impact on Dampier strain *A.franciscana* reproductive output or oviparity with the possible exception of increasing oviparity with low salinities. High salinities in particular do not appear to produce oviparity as

reported for other strains of *Artemia* (Davis 1979, Sorgeloos et al. 1986, Browne et al. 1992). It is likely that cysts produced in low salinity ponds remain in suspension until the brine density increase makes them buoyant enough to float leading to their accumulation in high salinity ponds. Further experiments on the reproductive response of *A.franciscana* to salinity could be undertaken from an academic viewpoint but from a practical viewpoint it does not appear that salinity has a great direct impact on reproductive output.

7.4.4 The reproductive response of *A.franciscana* to starvation and full sunlight. (Chapter 5.4).

Primarily oviparous animals captured from clear saltfield brine often became ovoviviparous in the laboratory (e.g. Chapter 5.3, Experiment 5.14). Two experiments were designed to determine whether the high light intensities experienced by the saltfield animals or the general lack of food in this environment influenced reproductive mode.

In Experiment 5.15 the reproductive response of *A.franciscana* to starvation was tested and all animals remained ovoviviparous until death.

In Experiment 5.16 the reproductive response of *A.franciscana* to food level and light intensity was tested and animals again remained ovoviviparous until death.

There was no indication in either experiment of a switch to oviparity when conditions became unfavourable as suggested by Lenz and Browne (1991) in their review. It is possible that conditions became unfavourable too rapidly. There is scope for similar experiments to be conducted in a less severe manner over a longer time period.

7.4.5 The reproductive response of *A.franciscana* to Oxygen stress and Iron in large scale outdoor culture.

A single experiment was conducted. This was based broadly on the work of Versichele and Sorgeloos (1980) but was conducted on a large scale, outdoors, and using nitrogen flushing to reduce oxygen levels rather than discontinuous aeration. Discontinuous aeration techniques were not effective in rapidly reducing oxygen levels in preliminary trials. The experiment was technically difficult to conduct and methods were developed on the basis of experience gained in three previously commenced and abandoned experiments. The experiment was inconclusive due to the fact that *Artemia* in all treatments were primarily oviparous but there was also high variability among replicates leading to low statistical power.

7.4.6 General Conclusion for reproductive test work

There were many practical outcomes achieved as a result of the test work summarised above. The screening systems successfully applied to the large-scale experimental pond described in Chapter 6 can, for example, be directly attributed to this work. There are, however, a number of areas warranting further study and the switching mechanism controlling reproductive mode, in particular, has still not been identified.

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APPENDIX 1

Composition of seawater and its concentrates (Baseggio, 1972)

Grams of ions per 1000 grams of brine as a function of brine density (22.2/15.6) g/1000 g of brine at 22.2° C

Sp. Gr.	ppt	Ca	SO_4	Mg Salts	Cl	K	Na	Total
1.0050	07.2	0.099	0.627	0.302	4.52	0.091	2.50	8.16
1.0100	14.4	0.179	1.140	0.548	8.20	0.164	4.53	14.79
1.0150	21.4	0.257	1.652	0.793	11.88	0.238	6.57	21.43
1.0200	28.4	0.335	2.163	1.037	15.51	0.311	8.58	27.99
1.0300	42.2	0.489	3.183	1.520	22.81	0.457	12.63	41.17
1.0400	55.8	0.641	4.199	2.000	30.04	0.601	16.64	54.22
1.0500	69.1	0.790	5.211	2.474	37.22	0.745	20.63	67.20
1.0600	82.1	0.937	6.220	2.944	44.35	0.888	24.61	80.10
1.0700	94.9	1.083	7.226	3.410	51.43	1.030	28.55	92.91
1.0800	107.4	1.226	8.229	3.872	58.47	1.171	32.48	105.65
1.0900	119.7	1.363	9.204	4.344	65.27	1.307	36.23	117.94
1.1000	131.8	1.256	9.686	4.844	72.91	1.460	40.51	130.92
1.1100	143.7	1.157	10.17	5.339	80.44	1.610	44.72	143.71
1.1200	155.4	1.063	10.64	5.828	87.85	1.759	48.85	156.29
1.1300	166.8	0.975	11.12	6.313	95.17	1.905	52.93	168.74
1.1400	178.1	0.892	11.59	6.793	102.4	2.050	56.95	181.03
1.1500	189.1	0.812	12.07	7.268	109.5	2.192	60.90	193.12
1.1600	200.0	0.735	12.54	7.739	116.5	2.332	64.78	205.03
1.1700	210.7	0.662	13.01	8.206	123.5	2.473	68.55	216.82
1,1800	221.2	0.591	13.48	8.668	130.4	2.611	72.50	228.70
1.1900	231.5	0.522	13.04	9.127	137.1	2.745	76.17	240.07
1,2000	241.7	0.456	14.41	9.581	143.8	2.879	80.06	251.68
1,2100	251.7	0.391	14.87	10.03	150.5	3.013	83.63	262.95
1,2200	261.5	0.325	16.33	11.40	155.9	3.405	84.97	272.71
1.2300	271.1	0.254	25.16	17.63	153.3	5.270	74.96	277.53
1,2400	280.6	0.194	33.17	25.53	151.9	7.029	65.83	282.85
1.2500	290.0	0.140	40.71	29.16	150.7	8.711	57.18	288.09
1.0247[1]	35.0	0.408	2.643	1.265	18.95	0.380	10.48	34.19
1.0897[2]	119.4	1.364	9.195	4.322	65.14	1.304	36.13	117.27
1.2185[3]	260.0	0.338	15.26	10.42	156.23	3.128	86.79	272.64
1.2450[4]	285.3	0.166	37.00	26.38	151.23	7.880	61.43	285.38

[1] Sea water from the open oceans.

[2] Density where $CaSO_4$ deposits

[3] Density where NaCl starts to precipitate.

[4] Density where bitterns is discarded.

APPENDIX 2 : Calibration of Dissolved Oxygen Meter

Calibrations were obtained by comparing meter readings with actual oxygen levels obtained from Winkler titrations as described in Strickland and Parsons (1968). Separate equations were obtained from a range of specific brine densities with oxygen levels manipulated via various combinations of heating, cooling and aeration. Equations were developed from the plotted graphs by Phillip Loh (Dampier Salt Ltd Chemical Engineer).
Y=Actual O_2 level, X=Meter Reading

Brine density	Equation	Correlation Coefficient (r)
1.0260g/mL	Y=1.1358+0.6135X	0.9997
1.0460g/mL	Y=2.0735+.4271X	0.9991
1.0580g/mL	Y=1.5726+.3901X	0.9988
1.0709g/mL	Y=2.152 +0.2174X	1.0
1.1556g/mL	Y=2.1372+0.0296X	0.95496

Overall Equation

$O_2(ppm) = 2.428 \times Density^{-7.653} \times Meter\ Reading^{0.5304}$

$r^2 = 0.967$

Equation not to be used for data outside the range used in the correlation

APPENDIX 3: Preliminary Experiment: Effects of Aeration and Shading on Growth of Microalgae in Outdoor Culture.

Culture mixing induces a fast movement of cells from the illuminated upper layer to the lower unilluminated layer and back and is one of the most basic requirements for high productivity in microalgal mass culture (Richmond, 1986a).

The effect of aeration and shading on the growth and dominance of microalgae present in brine of density 1.055 g/mLcollected from the salt field and fertilised were assessed under winter conditions in June 1989. The brine was not sterilised and contained a range of planktonic microalgae.

A series of 12 fibreglass tanks of capacity 1,000 L and of height 1 metre were filled with brine of density 1.055 g/mL collected from the salt field and fertilised at a level of 0.01 g "Thrive" fertiliser per litre. Combinations of aeration and shading were applied to the tanks in duplicate and one tank in each treatment had an additional 0.01 g "Thrive" fertiliser added on a weekly basis. The trial was an unreplicated range finding experiment. Five tanks were used for one treatment in an effort to determine variability between replicates.

Physical Conditions	**0.1g Thrive**	**0.1g Thrive/week**
1. Shaded/aerated	1 tank	1 tank
2. Shaded/unaerated	1 tank	1 tank
3. Full sun/aerated	1 tank	1 tank
4. Full sun/unaerated	5 tanks	1 tank

The tanks were shaded with 80% shade cloth and air delivered from an axial air blower through perforated PVC pipe. Brine temperatures were obtained from Maximum/Minimum Thermometers placed in each tank. Plankton was monitored daily and pH, brine density, light intensities at noon and dissolved reactive phosphate were monitored weekly.

Results

The only microalgae species to grow in any tank was *D.viridis* and this species only grew in aerated brine in full sunlight (Table 1).

Table 1: Range of Physico-Chemical Parameters Experienced During Trial and Final Mean Number of *D.viridis*/mL.

Treatment	**Temperature**	**pH**	**Density (g/mL)**	**Final PO_4 ppb**	***Dunaliella viridis*/mL**
1.Sh+A	12-22	7.76-8.11	1.0551-1.0662	487	0
2.Sh+A F	13-23	8.04-8.2	1.0554-1.0680	2207	0
3.Sh-A	13-22	7.95-8.36	1.0551-1.0577	310	0
4.Sh-A F	13-24	7.96-8.64	1.0552-1.0648	1550	0
5. S+A	12-25	7.99-8.24	1.0553-1.0718	16	$4400x10^3$
6. S+A F	12-24	8.06-8.55	1.0552-1.0707	1364	$1300x10^3$
7. S-A	11-26	7.95-8.91	1.0548-1.0667	22	0
8. S-A F	12-28	7.96-8.78	1.0552-1.0686	1209	0

Sh = Shaded = 2,067 lux at noon; s = Sun = 17,500 lux at noon; +A = Aerated; -A = Unaerated; F = Additional fertiliser added weekly.

APPENDIX 4 : Weight of *Dunaliella* and *Artemia* length/weight relationship.

WEIGHT OF DUNALIELLA FOOD SUPPLY

Culture containing 2,475,000 cells/mL was filtered through preweighed filter papers and rinsed with distilled water to remove all salt before being oven dried for 24 hours at 100°C. The papers were allowed to cool in a dessicator before reweighing. A consistant dry weight of 6.5×10^{-11}g/cell was obtained from four replicates and 7.03×10^{-11}g/cell from two others. The lower value of 6.5×10^{-11}g/cell was used in calculations.

LENGTH TO DRY WEIGHT RELATIONSHIP

This was determined by Reeve (1963) and published in graphic form. These data correspond to the following equation developed by P.Loh (unpublished) a Chemical Engineer with Dampier Salt.

$$\text{Dry Weight (g)} = 0.337 \times 8.967^{1/\text{length(mm)}} \times \text{length(mm)}^{3.314}$$
$$r^2 = 0.9981$$

For comparison a number of other Length/Dry Weight equations have been published.

1. A.Grobois, 1988. (Also using Reeves' data)

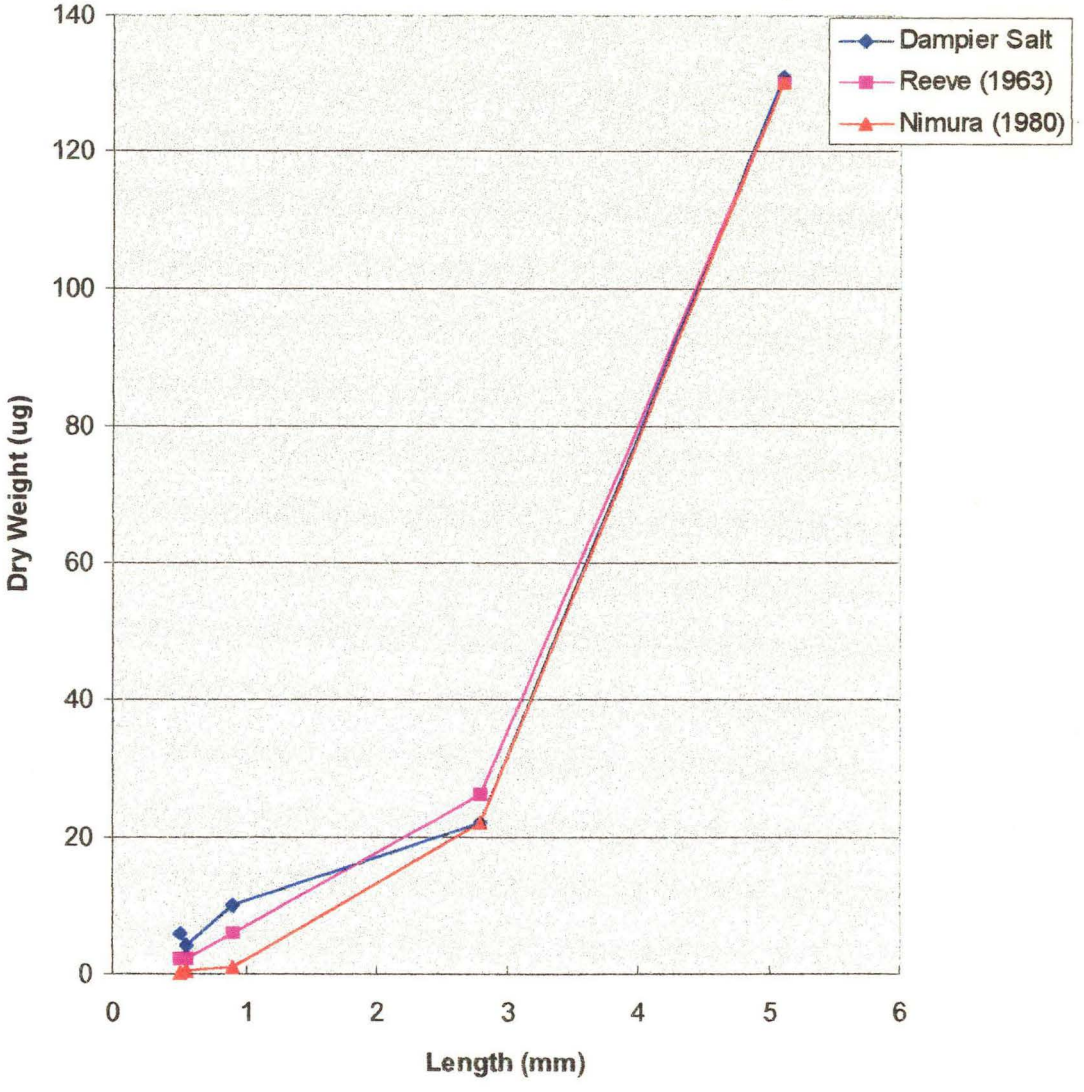
Length < 1.65 mm
Dry Weight(g) = length(mm)x0.49x10

Length > 1.65 mm
Dry Weight(g) = length(mm)x0.51x10

2. Nimura, 1980. (Using his own data)

$$\text{Dry Weight (ng)} = 857\text{L(mm)}^{3.00}$$

Spot checks on a variety of different sized *Artemia* collected from the Dampier Salt saltfield were used to verify these published data. The method involved carefully measuring a number (20 for small nauplii, 10 for large *Artemia*) of collected *Artemia* and washing them several times in distilled water. They were then placed on preweighed cavity slides and driedat 100°C for 24 hours. The slides were allowed to cool in a dessicator and weighed on a five place balance to obtain net dry weights of animals.



Comparison of Length/Weight relationships published by Reeve (1963) and Nimura (1980) compared to spot data for animals collected from the saltfield at Dampier.

APPENDIX 5: Composition of Soluble FertilisersTrialed in Section 3.2.

	Aquasol	Thrive	Zest	Cropspur
Nitrogen	0.23	0.31	0.23	0.24
Phosphorous	0.04	0.0457	0.04	0.04
Potassium	0.18	0.0871	0.18	0.18
Iron	0.0006	-	-	0.0002
Manganese	0.0015	0.0002	0.00135	0.0001
Copper	0.0006	0.00005	0.001	0.0001
Zinc	0.0005	0.0002	0.0002	0.0001
Boron	0.00011	0.00005	0.00037	0.0001
Molybdenum	0	0.00002	0	0

APPENDIX 6: FAILED MICROALGAE EXPERIMENTS

In addition to the experiments reported in Chapter 3 a large number of unreported microalgae experiments, particularly *T.suecica* experiments were commenced and abandoned for a variety of reasons.

Experiment 89/1(28/02/89): The effect of aeration and shading on the growth of *T.suecica* in 20 L outdoor aquaria. Abandoned due to dilution from rain after one week.

Experiment 89/2 (10/03/89): The effect of shading, aeration and weekly addition of fertiliser on the growth of *T.suecica* in 1,000 L outdoor tanks. Terminated after 7 days due to proliferation of benthic cyanobacteria in all tanks.

Experiment 89/4 (23/03/89): The effect of macronutrient concentrations on the growth of *T.suecica* in 1,000 L outdoor tanks. Abandoned after two weeks due to cyanobacteria bloom.

Experiment 89/6 (6/4/89): To test the effect of microalgal succession in unsterilised field brine compared to sterilised (autoclaved) brines inoculated with pure cultures of *D.viridis* and *T.suecica* in 250 mL beakers in the laboratory. Abandoned after 21 days when no algae grew in the sterilised media.

Experiment 89/7 (13/04/89): The effect of shade and aeration on the growth of *T.suecica* in 1,000 L outdoor tanks. Abandoned due to cyclone but *T.suecica* was growing well in full sun unaerated brine at this stage.

Experiment 89/9 (12/05/89): The effect of vitamins on the growth of *T.suecica* in 250 mLlaboratory culture. Eventually abandoned when nothing grew.

Experiment 89/11 (20/05/89): The effect of shade and aeration on the growth of *T.suecica* in 1,000 L outdoor tanks. Abandoned due to rain.

Experiment 89/12 (21/06/89): The effect of various media on the growth of *T.suecica* in 250 mLlaboratory culture. Nothing grew.

Experiment 89/14 (7/7/89): The effect of brine density on the growth of *T.suecica* in 250 mL laboratory culture. Nothing grew.

Experiment 89/15 (9/8/89): The effect of Thrive concentrations (0.005-0.1 g/L) on the growth of *D.viridis* in the laboratory. It did not grow.

Experiment 89/17 (12/09/89): Effect of shade and aeration on the growth of *D.viridis* in 1,000 L outdoor culture. Did not grow.

Experiment 89/18 (13/09/89): Repeat of 89/17. Still did not grow.

Experiment 89/19 (14/09/89): The growth of *D.viridis* in a range of media in 250mL laboratory culture. It would not grow.

Experiment 89/20 (23/10/89): The effect of aeration and shading on the growth of *D.viridis* in 1,000 L outdoor culture. Nothing grew so abandoned.

Experiment 90/1 (22/1/90): The effect of aeration on the growth of microalgae in unsterilised field brines in 1,000 L outdoor culture. Highly variable results and not useful.

Experiment 90/2 (27/02/90): A repeat of 90/1 abandoned due to rain.

These experiments indicate the vagaries of biological production in an extreme climate. Most of the failed algal trials involved species other than *D.salina*, the preferred species for large-scale production in the Dampier saltfield.